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Effect of vegetable oil on the degradation of a pesticide mixture in biobeds

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ABSTRACT

Pesticides are used to control diseases, undesired vegetation and insects in forestry, gardening and agriculture. In the environment, pesticides are generally believed to originate from a diffuse source, such as spray drift of pesticides during application, or a point source, such as spills during refill and cleaning of spraying equipment. Point sources are easier to eliminate than diffuse sources and one method to reduce the leaching from point sources is to use biobeds as bioprophylaxis.

The Swedish biobed was developed by Torstensson and Castillo in 199X (KB) and is constructed with locally available constituents, such as peat, wheat straw and soil. The main advantages of the biobed is that it is very simple and cheap, but still very effective. On sites where vegetable oil have been spilled on the biobed, e.g on farms producing rapeseed oil, both increased activity of lignin degrading microorganisms and increased pesticide degradation have been observed.

The lignin degrading microorganisms produce unspecific extracellular enzymes, which also are able to degrade recalcitrant molecules such as organic pollutants. The most important ligninolytic enzymes, currently known, are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. Pure MnP have been found to be able to initiate lipid peroxidation. Due to recent findings that vegetable oil, which has high levels of unsaturated fatty acids, is increasing the effect of lignin degrading microorganisms, addition of oil is proposed to further increase the biobed efficiency.

Since the crop is determining which pesticide to be used, pesticides are often encountered in mixtures in the environment. For this purpose five selected pesticides will represent the mixture of pesticides. The selected pesticides are bentazone (BZ), metabenzthiazuron (MBTZ), isoproturon (IP), terbutylazine (TBA) and chlorpyrifos (CLP).

The main objectives of this study were:

I. To investigate whether the addition of rapeseed oil to the biomix (mixture of peat, soil and straw used in a biobed) enhanced the degradation rate of pesticides (BZ, MBTZ, IP, TBA and CLP).

II. To investigate whether a potential increase in degradation, of the same pesticide mixture, was explained by a MnP-mediated lipid peroxidation process.

The experiment was set up to study whether the addition of oil can have a positive effect on degradation of a mixture of pesticides in a biomix and in soil. Enzymatic activity and microbial respiration were followed during the incubation period.

The degradation of the pesticides by pure MnP in the presence of oil was tested *in vitro*. The experiment was prepared for each pesticide separate and a control without any pesticide added, in total six experimental setups.

The main results from these studies are summarized below:

- The effect from the addition of oil varies from promoting degradation to inhibiting degradation depending on pesticide. The lack of correlation with phenoloxidase activity could indicate that degradation mechanisms other than ligninolytic enzymes were prevailing or even dominant in this study.

- The microbial respiration was higher in biomix than in soils. The addition of oil had an inhibitory effect in the biomix.

- The phenoloxidase activity was higher in the biomix compared to the soil. The addition of oil did not increase the enzymatic activity.

- In the *in vitro* experiments the addition of oil enhanced the degradation of IP by MnP, but no positive effect was observed for CLP, TBA, BZ and MBTZ. However, no enhancement of IP degradation was obtained in the biomix in the presence of rapeseed oil.

- The initial method for the HPLC analysis was developed so all five pesticides could be detected within a reasonable time frame. However, with the oil-fractions peaking around MTBZ and BZ, and the fact that CLP had to be analysed separately, a new method also for the other four pesticides need to be developed for further studies. The new method for the four pesticides should separate the peaks around BZ and MBTZ further and not take CLP into account.

- The interference peak in the chromatograms which increased over the incubation time could a product from vegetable oil being degraded in presence of of MnP could possibly indicate lipid.

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1. INTRODUCTION

Pesticides are used to control diseases, undesired vegetation and insects in forestry, gardening and agriculture. In the environment, pesticides are generally believed to originate from a diffuse source, such as spray drift of pesticides during application, or a point source, such as spills during refill and cleaning of spraying equipment.

To protect sensitive recipients in environment, such as surface waters and groundwater, environmental samples are continuously collected to compare levels in the environment to established guideline values to assess the risk these pesticides poses to human and environmental recipients. The European groundwater directive proposed a generic guideline value for all pesticides of 0.1 µg/l. The Swedish pesticide database contained 8200 water samples in 2005, out of which 39% contained traces of pesticides. Despite the number of samples with high concentrations of pesticides has decreased since the 1970's, findings of samples with pesticide concentration over 0.1 µg/l are still common (Adielsson, Törnquist et al. 2006).

Pesticides can reach the non-intended recipients through diffuse sources, e.g. spray drift during application, or point sources, e.g spills of pesticides during refill or cleaning of application equipment (Castillo, Torstensson et al. 2008). Due to water access many farmers clean their equipment at the farm, and often at the same spot. If these sites also have high sand and gravel content, which decreases the retention time for pesticides, the risk for groundwater contamination is apparent (Castillo and Torstensson 2007). Point sources are easier to eliminate than diffuse sources and one method to reduce the leaching from point sources is to use biobeds as bioprophylaxis.

The Swedish biobed was developed by Torstensson and Castillo (1997) and is constructed with locally available constituents, such as peat, wheat straw and soil. The main advantages of the biobed is that it is very simple and cheap, but still very effective (Castillo, Torstensson et al. 2008). The high content of lignin in straw is favourable for the ligninolytic fungi, which are able to degrade complex organic structures such as lignin and other recalcitrant organic compounds. Even though the biobed is efficient in degrading pesticides, the degree of degradation efficiency varies between pesticides. To increase the degradation it is very important to improve the function of the biobed.

On sites where vegetable oil have been spilled on the biobed, e.g on farms producing rapeseed oil, both increased activity of lignin degrading microorganisms and increased pesticide degradation have been observed (Castillo, Torstensson et al. 2008).

2. BACKGROUND

2.1. The biobed

The Swedish biobed is typically installed in a 60 cm deep excavation with a 10 cm clay layer in the bottom (see Figure 2-1) overlain by the biomix that is composed of soil, lignin rich straw and peat. The clay layer increases the water retention time which consequently increases the moisture in the biobed and minimises the leaching of particle bound pesticides. The typical biomix contains peat (*Sphagnum*, no nitrogen amended), soil and lignin rich straw and a grass cover on top to regulate moisture by evapotranspiration. The grass cover can also indicate hot spots of pesticide spillages. Soil is providing increased sorption capacity, and also a microflora. Also the peat is increasing the binding sites as well as controlling soil moisture and retaining a low pH necessary for growth of ligninolytic fungi, and the straw contributes with microorganisms able to degrade lignin and other recalcitrant substances. The use of peat without nitrogen amendment is recommended since the production of lignin degrading enzymes by the microorganisms is inhibited by high nitrogen content (Castillo 1997; Torstensson 1997; Torstensson and Castillo 1997; Castillo and Torstensson 2007). Since the aim with the Swedish biobed is to construct a cheap bioprophylactic method, the biobed should be constructed from locally produced constituents. All constituents mentioned here are commonly available in Sweden.

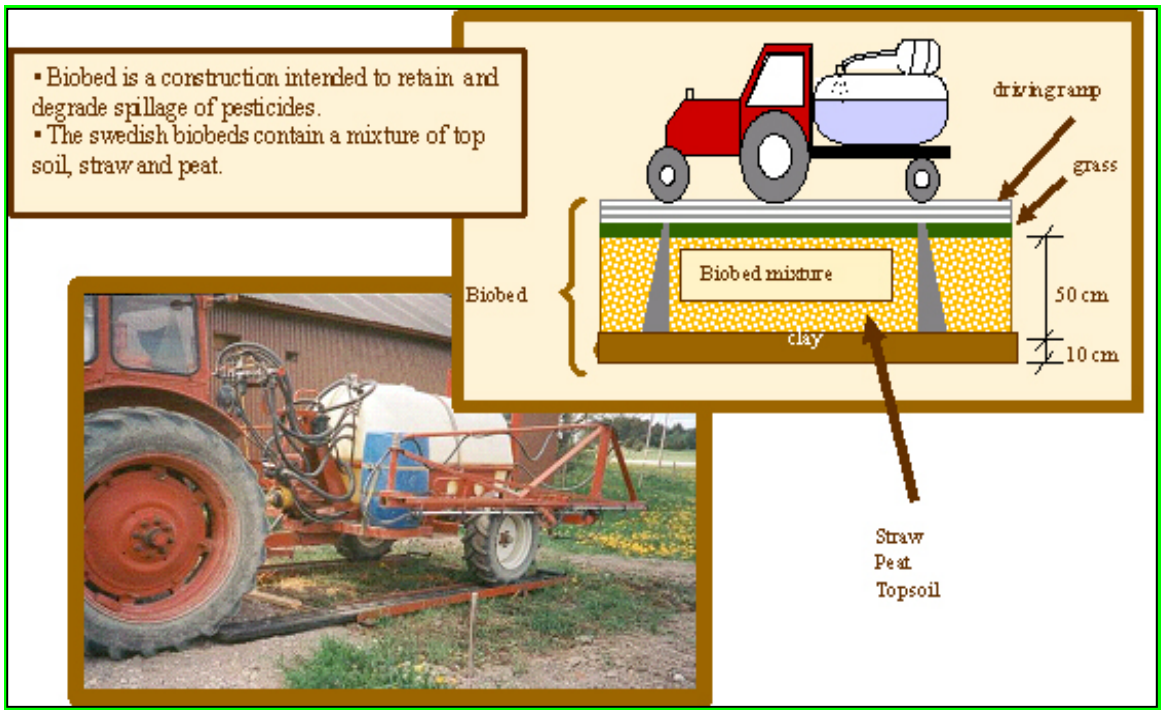


Figure 2-1 A schematic picture of the biobed and a photo of a biobed in use (Castillo and Torstensson 2007).

2.2. Degradation of pesticides by ligninolytic enzymes

The lignin degrading microorganisms produce extracellular enzymes, unspecific as regards to their target compounds. This property enables the enzymes to degrade recalcitrant molecules such as organic pollutants (Novotny, Svobodova et al. 2004). The most important ligninolytic enzymes, currently known, are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. In early studies, LiP was regarded as the main ligninolytic enzyme due to the high oxidation potential and its ability to oxidise even non-phenolic parts of the lignin. In contrast to LiP, MnP has a lower oxidation potential and does not oxidise non-phenolic compounds (Kapich, Prior et al. 2005; Lisov, Leontievsky et al. 2007). Even though many lignin degrading fungi produce LiP, some very important fungi such as *Ceriporiopsis subvermispora* do not. For a long time, it was assumed that Basidiomycota were the only phyla which could degrade lignin, but with time the lignin degrading ability has also been discovered in some strains of Ascomycota and in bacteria such as Actinomycetes (Kirk and Farrell 1987).

2.2.1. Manganese Peroxidase (MnP)

It is now known that MnP is a key enzyme in the degradation of recalcitrant substances by fungi. The enzymatic cycle of MnP (illustrated in Figure 2-2) is initiated by a two electron oxidation of the native enzyme (MnP N) to MnP Compound I (MnP I) by H_2O_2 (produced by the fungi). MnP I can oxidise Mn(II) to Mn(III) by a one electron transfer and be reduced to MnP Compound II (MnP II). Since Mn(III) is an unstable ion, it is chelated by an organic acid e.g. oxalate (also produced by the fungi). The oxidation of a mediator is what differs MnP from other peroxidases (Mester and Tien 2000). The first step, reduction of MnP I to MnP II can oxidise other molecules than manganese, but the oxidation of manganese is the dominating process. The second reduction of MnP, the reduction of MnP II to native MnP is only oxidising the manganese ion. If there is an excess concentration of H_2O_2 MnP II will be oxidised to MnP compound III (MnP III), a non-reactive species of MnP (Hofrichter 2002). The MnP-producing microorganisms are self sufficient in H_2O_2 production by oxidising reduced substrates like NAD(P), glutathione, dithiothreitol and dihydroxymaleic acid (Castillo, Stenström et al. 1993).

The chelated manganese ion is also much smaller compared to the enzyme molecule, and because of its small size it can oxidise the lignin compound in remote confined areas where the enzyme cannot enter.

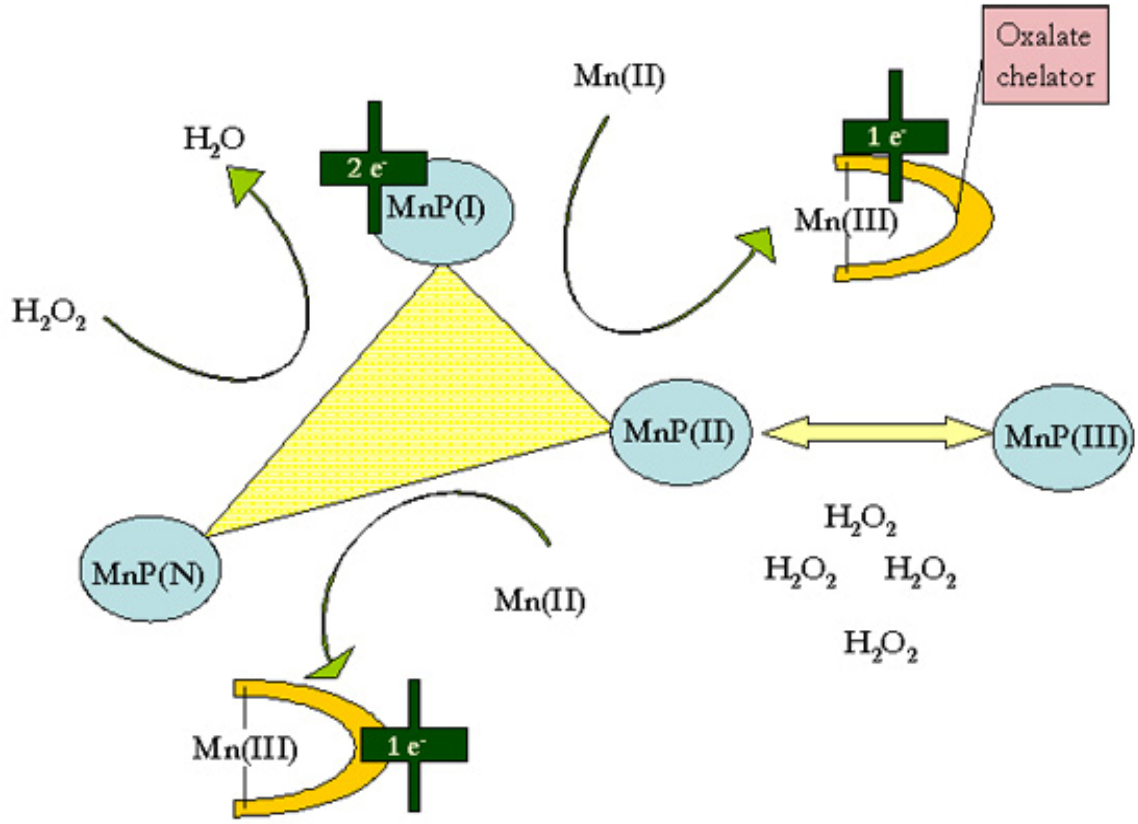


Figure 2-2 A schematic sketch of the enzymatic cycle of MnP; where MnP (N) is the native enzymatic compound, MnP(I) is undergoing a two electron oxidation, MnP(II) oxidised one step, and MnP(III) is a non-reactive state of the enzyme when there is an excess of H_2O_2 Adapted from Hofrichter, (Hofrichter 2002).

The oxidised chelated manganese ion oxidises the phenolic part of lignin compounds, or similar structures, by a one electron transfer. This creates a very reactive cation radical that undergoes various chemical reactions and the initial lignin compound is degraded (Castillo, Ander et al. 2000; Mester and Tien 2000). The mechanisms of LiP are similar, but LiP is often oxidising the substrate directly LiP can also oxidise the target compound via oxidation of veratryl acohol as well. MnP can oxidise the phenolic compounds of lignin, but fail to oxidise the non-phenolic ones. However, it is known that non-phenolic lignin can be degraded with only MnP present. It has been suggested that lipid peroxidation can be induced by MnP and in that way also oxidise non-phenolic compounds. The cation radical created in the enzymatic cycle can initiate a lipid peroxidation sequence in presence of unsaturated fatty acids (Bao, Fukushima et al. 1994).

2.2.2. Lipid peroxidation

The lipid peroxidation is an oxygen consuming chain reaction (Kapich, Hofrichter et al. 1999) producing a lipid peroxyl radical. The initiating radical could originate from a photoreaction or enzymatic activities (Kapich, Hofrichter et al. 1999). Pure MnP have been found to be able to initiate lipid peroxidation (Bao, Fukushima et al. 1994). Theoretically the same manganese ion could be oxidised over and over again in the enzymatic cycle and create a new lipid radical each time.

The process of lipid peroxidation in degradation of recalcitrant matter is complex and yet not fully investigated. However, the lipid peroxidation is targeting the unsaturated fatty acids, which contain high amounts of energy compared to hydrogen in a saturated fatty acid. The higher degree of unsaturation the higher is the rate of lipid peroxidation. Due to the high amount of unsaturated fatty acids, rapeseed oil is suggested to be a suitable source of unsaturated fatty acids. Rapeseed oil contains low amounts of saturated fatty acids (6.8 %), the content of polyunsaturated fatty acids is slightly higher (30.2 %) and the amount of monounsaturated fatty acids more than 50 % of the fatty acids. (58.6 %) (SLV, 2007).

Due to recent findings that vegetable oil is increasing the production of lignin degrading microorganisms, addition of oil is proposed to further increase the biobed efficiency. Oil also increases the pesticide solubility, increasing the amount of pesticides accessible to microorganisms (Kapich, Hofrichter et al. 1999). It could also be an extra source of carbon and thereby stimulate production of biosurfactants (Castillo and Torstensson 2007; Pizzul, Castillo et al. 2007).

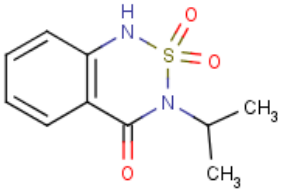
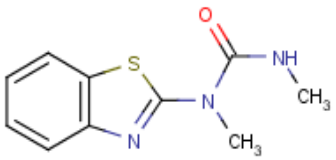
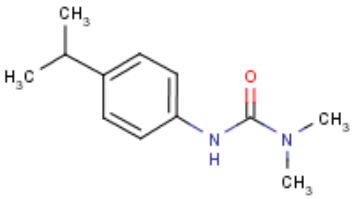
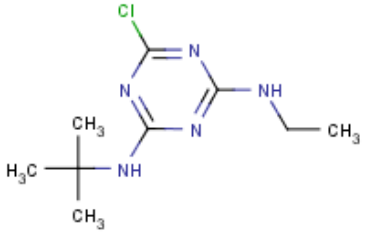
The increased degradation from the addition of vegetable oil has been attributed to the lipid peroxidation process and has also been verified for the degradation of some polyaromatic hydrocarbons such as benzo(a)pyrene (Pizzul, Castillo et al. 2007).

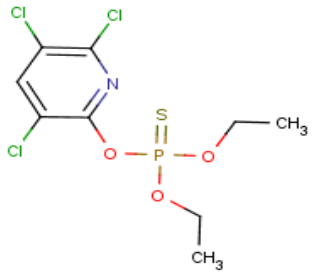
2.3. Pesticide mixtures

Since the crop is determining which pesticide to be used, pesticides are often encountered in mixtures in the environment. The selected pesticides are bentazone (BZ), metabenzthiazuron (MBTZ), isoproturon (IP), terbutylazine (TBA) and chlorpyrifos (CLP). Degradation in biobeds of all these pesticides has been studied, in varying extent and with varying results, however not in mixtures (Castillo, Ander et al. 2000; Coppola, Castillo et al. 2006; Castillo 2007; de Wilde, Spanoghe et al. 2007). The basic structure

and lipophilic characteristics of these pesticides are presented in Table 2-1. The higher Log P and the lower water solubility, the more lipophilic the compound. All of the pesticides have a log P > 0, which indicates that all of these pesticides are more lipophilic, fat soluble, than hydrophilic. All pesticides in this study have recently been encountered in Swedish ground and surface waters in concentrations up to 280 µg/l (bentazone). Four of them are herbicides and one, chlorpyrifos, is an insecticide. Bentazone is commonly used for ley and is still in use at present date (KEMI 2010). Isoproturon is allowed in Sweden but only the former is still being applied (KEMI 2010). Terbutylazine, Methabenzthiazuron and the organophosphate chloropyrifos were banned in 2003, 2005 and 2008 respectively (KEMI 2010).

Table 2-1 The basic characteristics of some commonly encountered pesticides in the environment with varying properties (TOXNET 2010).

Compound	**Structure**	**Water solubility**	**Log P (octanol –water)**
Bentazone		500 mg/L @ 20 ºC	0.77 pH 5 -0.46 pH 7 (The Pesticide Manual)
Metabenzthiazuron		59 mg/L @ 20 ºC	2.64
Isoproturon		65 mg/L @ 22 ºC	2.87
Terbutylazine		8.5 mg/L @ 20 ºC	3.21

Compound	Structure	Water solubility	Log P (octanol –water)
Chlorpyrifos		1.12 mg/L @ 24º C	4.96

2.4. Objectives

The main objectives of this study were:

I. To investigate whether the addition of rapeseed oil to the biomix (mixture of peat, soil and straw used in a biobed) enhanced the degradation rate of pesticides (bentazone, chlorpyrifos, isoproturon, methabenzathiazuron and terbutylazine).

II. To investigate whether a potential increase in degradation, of the same pesticide mixture, was explained by a MnP mediated-lipid peroxidation process.

3. METHODS AND MATERIALS

3.1. Chemicals

For this purpose five selected pesticides will represent the mixture of pesticides. Bentazon (BZ), metabenzthiazuron (MBTZ), isoproturon (IP), terbuthylazine (TBA), chlorpyrifos (CLP) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Manganese peroxidise from *Nematoloma frowardii* was provided by JenaBios. 3-(dimethylamino)benzoic acid (DMAB) and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were supplied by Sigma-Aldrich (Steinhelm, Germany). Tween 20 was supplied by Merck (Darmstadt, Germany).

3.2. Effect of oil on the degradation of pesticides in biomix and soil

The experiment was set up to study whether the addition of oil can have a positive effect on degradation of a mixture of pesticides in a biomix and in soil. Enzymatic activity and microbial respiration were followed during the incubation period.

3.2.1. Soil and biomix

One week prior to experimental start-up, biomixture and soil control was prepared. Soil used for this experiment was agricultural top soil from Ulleråker, 5 km south of Uppsala and was used as a constituent in the biomixture and as soil control. The soil was sieved through a 2 mm sieve. Commercial peat (sphagnum) was thoroughly parted and added to the biomixture. For this experiment, chopped wheat straw (2 mm) was used for the biomixture. The volume proportion of the constituents for the experiment was one part of soil, one part of peat and two parts of straw.

Before start up of the experiments, initial water content, loss on ignition, water holding capacity (WHC) and pH were measured. The water content was adjusted to 70% of the WHC in the treatments (soil and biomix with and without oil). The soil and biomix were incubated at 30°C one week prior to experimental start up. During the whole experiment the water content was adjusted to 70% of the WHC.

3.2.2. Experimental set up.

The biomix (8 g ± 0.02, 6.3 % of weight; %-W) and soil (20 g ± 0.02, 7.2 %-W) was portioned into plastic 100 ml vials, whereupon oil (0.5%-W) and pesticides were added to respective treatment on a weight basis and water content was adjusted to 70% of WHC. A

1000 ppm mixture of BZ, MBTZ, IP, TBA and CLP (200 ppm of each pesticide) in methanol solution was used (from here on referred to as pesticides). In total four different treatments were prepared in triplicates, soil without oil added (S – O), soil with oil added (S + O), biomix with no oil added (BM - O) and biomix with oil added (BM+O).The soil and biomix were kept at 30°C throughout the experiment. From here on, one sample refers to the content of one 100 ml plastic vial.

Three samples were collected from each treatment on day 0, in addition to the samples mentioned above. These samples were followed during 70 days of the experiment. Respiration experiment was set up in an air sealed glass jar with one sample of soil (the same for the whole experiment) and vials with 4 ml of 0.2 M NaOH.

3.2.3. Sampling

Each sample was labelled with an identification number prior to experimental start up. At each sampling occasion the samples to be collected were pre-determined by randomly selecting a sample number. Samples were collected at day 0 (application day of pesticides), day 10, day 20, day 31, day 51 and finally on day 70. At each occasion, nine samples from each treatment (S – O, S + O, BM – O and BM + O) were collected for analysis of pesticide (3 samples), measurement of phenoloxidase activity (3 samples) and measurements of pH, dry weight and loss on ignition (3 samples)

Samples for analysis of phenoloxidase activity and pesticide dissipation were kept in -20ºC freezer until analysis. Samples were analysed immediately for pH, dry matter and loss on ignition.

3.2.4. Microbial respiration in soil and biomix in the presence of rapeseed oil

Respiration was measured throughout the experiment by trapping CO_2 in vials with NaOH. The vials with NaOH were changed regularly to avoid complete saturation of the NaOH. The amount of CO_2 was determined by titrating the remaining alkali with 0.1 M HCl after precipitation of the carbonate with 0.1 M $BaCl_2$.

To analyse CO_2 as a marker for respiration, 2 ml of NaOH were added to 4 ml of 0.1 M $BaCl_2$ and titrated with 0.1 M HCl to pH 8.3. The amount of HCl was related to the amount of CO_2 per gram soil/biomix produced by the respiration of micro organisms by the equation below.

$$mg_{CO_2} = \frac{n_{HCl}(V_{HClref} - V_{HClsamp}) * MW_{CO2} * \#vials}{weight_{samp}(g_{solids})}$$

Where n_{HCl} was 0.2 M, MW_{CO2} was 44, $V_{HCl\ ref}$ was background from NaOH (ml) measured at each sampling occasion and V_{HCl} was based on the result from the titration (ml).

3.2.5. Phenoloxidase activity

Phenoloxidase activity was measured with the MBTH/DMAB assay (Castillo, Stenström et al. 1993). The basic principles of the assay is that the enzyme catalyses the formation of a deep purple compound when H_2O_2, $MnSO_4$, DMAB and MBTH are present. Since this compound has an adsorption peak at 590 nm, the activity of MnP can easily be quantified with a spectrophotometer.

To let the samples thaw, they were kept in a + 4ºC room for 3-10 days before measuring the activities and degradation.

Lactate-succinate buffer (0.1 M, pH 4.5) was mixed with the sample (1:2 g:ml for soil and 1:3 g:ml for biomixture) in a Duran glass bottle, and shaken for 1 hour at 100 rpm. The liquid phase was transferred to a Teadlar bag and homogenized in a Stomacher for 30 sec at normal speed. The supernatant was then collected, centrifuged at 4000 rpm for 20 min, filtered through a 0.45 µm filter and mixed with the reagents in a plastic cuvette as shown in Table 3-1.

Table 3-1 Reagent mixture for measurement of phenoloxidase activity with MBTH/DMAB assay. Reagents are presented in the order added to the spectrophotometric cell. H_2O_2 was added to start the reaction.

Reagents	**Volume**
Succinate-lacctate buffer 0.1 M, pH 4.5	1060 µl
DMAB, 6.6 mM	300 µl
MBTH, 1.4 mM	100 µl
$MnSO_4$, 20 mM	30 µl
Sample extract	500 µl
H_2O_2, 10 mM	10 µl
Total Volume	2000 µl

All reagents (except H_2O_2 and sample) were heated to 30°C prior to mixing. At time 0, the H_2O_2 was added to start the reaction and accumulated absorbance was registered for the following 5 min. Phenoloxidase activity is the rate of measured accumulated absorbance.

3.2.6. Pesticides analysis

Based on the three replicates collected at day 0 from each treatment, the initial extraction efficiency could be calculated since a known amount was added to each sample.

The pesticides were extracted from a sample with 1:2.5 (soil) or 1:4 (biomix) ml methanol, and shaken over night (16 h) at 200 rpm. The sample was centrifuged at 5000 rpm for 10 min, and the supernatant was transferred to High Performance Liquid Chromatograph (HPLC) vials and centrifuged again (5 min, 5000 rpm) before analysis.

To let the samples thaw, they were kept in a + 4ºC room for 3-10 days before measuring the activities and degradation.

3.3. Degradation of pesticides by pure MnP with vegetable oil

The degradation of the pesticides by pure MnP in the presence of oil was tested *in vitro*. In this experiment, pure MnP (hydrogen-peroxide oxidoreductase EC 1.11.1.13) from *Nematoloma frowardii* was used. The experiment with controls was set up according to Table 3-2. For this experiment pesticides were prepared in five individual 200 ppm methanol solutions to avoid deterioration of the pesticide during storage.

Table 3-2 Experimental set up for measuring in vitro dissipation of pesticides by pure manganese peroxidase (MnP) with presence of rapeseed oil. Each treatment was made in three replicates.

		A. MnP + oil	B. Oil	C. Control 1	D. Control 2	E. Control 3	F. Control 4
1	Oil (mg)	20	20	0	0	20	20
2	NaAcetate Buffer, 50mM pH 4.5 (µl)	1500	1550	1550	1500	1560	1800
3	Tween 20 10% (µl)	100	100	100	100	100	100
4	$MnSO_4$ 50 mM (µl)	40	40	40	40	40	40
5	Pesticides Mix. (25 ppm) (µl)	300	300	300	300	300	0
6	H_2O_2 10 mM	10	10	10	10	0	10
7	MnP (6 U/µl) (µl)	50	0	0	50	0	50
	Total volume (excluding oil)	2000	2000	2000	2000	2000	2000

The experiment presented above was prepared for each pesticide separate and a control without any pesticide added, in total six experimental setups. The reagents were mixed in 2 ml glass vials (sterilised in 550°C and the plastic caps were autoclaved). Except for the H_2O_2, all reagents were prepared beforehand and stored in fridge until mixing. To avoid

decomposition, the H_2O_2 was prepared in a bottle covered with aluminium folium just before the mixing of the treatments.

Each treatment was mixed and sampled immediately. The vials were placed with loosely tightened caps in a water bath at 37°C and shaken at 150 rpm and samples were taken immediately after the addition of MnP and at the same time daily for 7 days. The sample (200 µl) was added to an Eppendorf tube with 300µl of methanol to stop the reaction, and placed on ice during sampling. The pesticide content was analysed instantly (see section 3.4). After sampling on day 4, 50 µl of MnP was added to treatment A, D and F, and 50 µl of Na acetate buffer to treatment B, C and E.

3.4. HPLC analysis of pesticides

To estimate the dissipation of pesticides, the samples were analysed by High Performance Liquid Chromatography (HPLC).

3.4.1. Description of HPLC method

The column used in this method was Zorbax SB-C18, 150*4.6 mm i.d. at 40°C with a column flow of 1.000 ml/min in the quartenary pump. Injection volume was 5 µl of sample in methanol solution. Two solvents were used, the pump with 20 mM KH_2PO_4 (pH 2.5) was run at 100 % during the entire method and the port for the second solvent, acetonitril, was run from the 17^{th} until the 23^{rd} minute. The increase in acetonitril flow causes some change in pressure, which changes the retention time for chlorpyrifos.

The maximal flow ramp was 100.00 ml/min^2, compressibility was $100*10^{-6}$/bar, and primary channel and minimal stroke was set to auto. All agilent contacts (four) were open initially and the first one was closed at time 0.02 minutes. The isocratic pump had no acetonitril and only KH_2PO_4 (pH 2.5) between the 17^{th} and 23^{rd} minute and a maximum pressure at 400, otherwise identical to the quartenary pump. The total run time for this method was 22 min.

Since the pressure changes when altering the acetonitril/KH2PO4 proportions, which causes high fluctuations of the retention time for chlorpyrifos, a separate method was used for measure chlorpyrifos. In this method, the acetonitril only increased to 80% between 16 min and 20 min. The total run time was 20 min.

The wavelength detector has a peakwidth of 0.1 min and a wavelength at 230 nm (190 - 400 nm), attenuation analog out was 1000 mAU, zero offset was 5 %, no store additionally and only pre run balancing.

Due to complex chromatograms with high background activities and risk for interferences, the amount was derived from the height of the peak.

3.5. Statistical analysis

t-tests were performed with Microsoft Excel with a two-tailed distribution to establish whether the treatments differed from each other, alfa was set to 0.05. The data were assumed to be normally distributed and not related, unpaired and with the same variance.

4. RESULTS

4.1. Effect of oil on the degradation of pesticides in biomix and soil

Even though the pH for both biomix and soil only varies between 6.1 and 6.9 (see Figure 4-1) there was a generally lower pH in the treatments with biomix. However, there seems to be no clear effect from the oil added on pH measured neither in the soil nor the biomix.

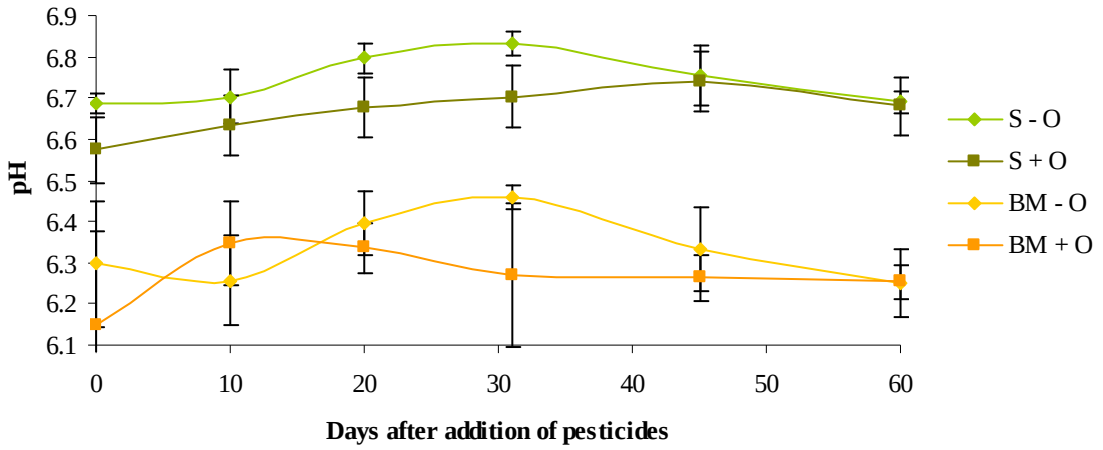


Figure 4-1 pH in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V). The samples contained a mixture of BZ, MBTZ, IP, TBA and CLP (20 ppm of each pesticide) and were incubated at 30C. Mean values +- SD, n=3.

4.2. Respiration

In general biomix samples had a higher respiration rate compared to the soil samples (Figure 4-2). There was no significant difference between the two soil treatments. A higher respiration rate was observed in biomix without oil (BM - O) towards the end of the experiment whereas a decreasing respiration was observed in biomix with oil (BM + O). Further, the biomix treatments have similar respiration rates until day 24 after which the respiration rate in the treatment with oil was lower compared to the treatment without. Due to compromised replicate, only two replicates were used for BM + O.

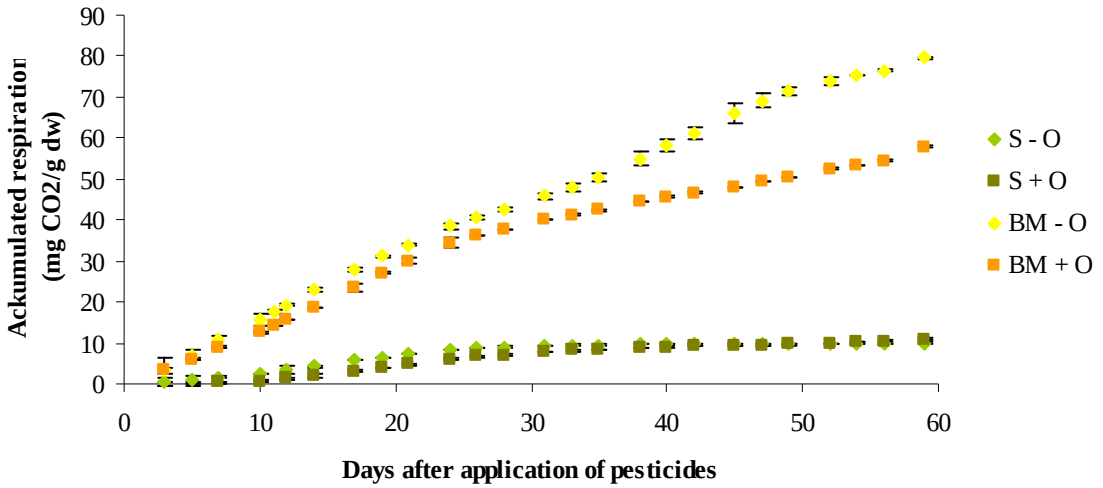


Figure 4-2 The microbial respiration expressed as mg CO2/g DW) in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V). The samples contained a mixture of BZ, MBTZ, IP, TBA and CLP (20 ppm of each pesticide) and were incubated at 30C. Mean values +- SD, n=3, except for BM+O where n=2.

4.3. Phenoloxidase activity

There was a seemingly higher phenoloxidase activity in treatments with biomix, compared to the soil treatments (see Figure 4-3). Figure 4-3 shows phenoloxidase activity corrected with results from blank samples with no extract from soil or biomix added. Due to very low phenoloxidase activity in the soil treatments the activity sometimes appears negative, since the values were corrected for background activity in the mixtures where no sample extract was added (Table 3-1). Also, due to lack of trends in phenoloxidase activity, no statistics was performed to distinguish significant differences between the treatments. However the results indicate a higher phenoloxidase activity in the soil with addition of oil.

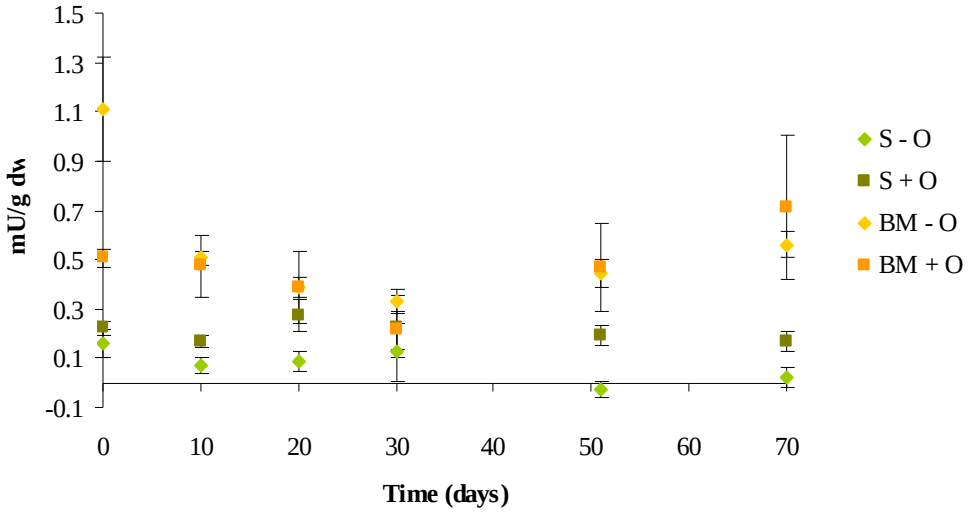


Figure 4-3 Phenoloxidase activity over time in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) and were incubated at 30°C. Values are presented as average +- standard deviation (n=3)

4.4. Degradation in biomix and soil

In the following sections the results from the degradation experiment are presented individually for each pesticide. Selected graphs are presented in the Results section of the report, for complete time graphs for all pesticides and treatments, see Appendix A. Although pesticides were added as a mixture to soil and biomix, each pesticide is presented individually. Due to compromised analytical results, samples from day 10 have been regarded as non-relevant outliers.

4.4.1. Extraction efficiency results

The extraction efficiency was measured initially and results were present in Table 4-1. The extraction efficiency is a measure of how much of a known amount added pesticide are extracted with the used extraction methods.

Table 4-1 Results for extraction efficiency in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) and were incubated at 30C. Mean values +- SD, n=3.

	S - O	S + O	BM - O	BM + O
Bentazon	89,9	89,0	95,0	92,6
Methabenzthiazuron	97,3	98,3	108,6	107,5
Isoproturon	99,1	100,3	94,6	95,8
Terbutylazine	99,9	100,5	95,2	95,8
Chlorpyrifos	81,8	75,9	76,3	76,4

The extraction efficiency was generally between 89 and 100 %, with the exception for CLP, where an extraction efficiency between 75 – 82 % was calculated. In biomix, an extraction efficiency over 100 % was calculated for MBTZ.

Bentazone (BZ)

The final dissipation of BZ (Figure 4-4) in biomix was significantly higher compared to soil ($p<0.05$). However, there was no statistical difference in dissipation between treatments with and without oil, neither for soil nor for biomix.

A lag phase is observed until day 20, from when the majority of the dissipation in biomix occurs (Table 4-2). The dissipation rates are calculated from day 20 until the end of the experiment.

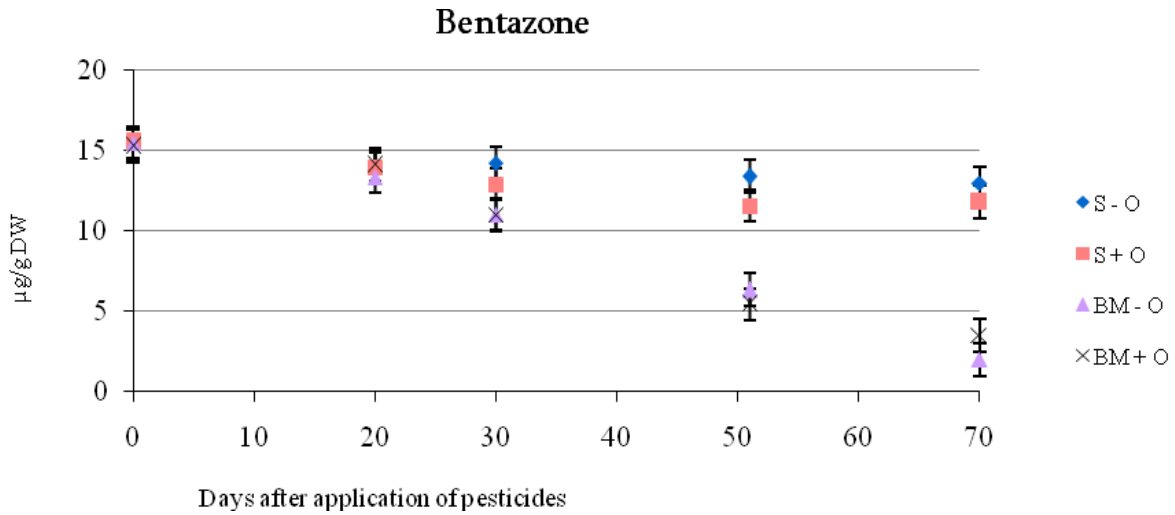


Figure 4-4 Concentration of bentazon as mean values +- SD, n=3 in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) that were incubated at 30ºC.

Table 4-2 Average dissipation rate of bentazone as mean values, n=3 in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) that were incubated at 30ºC.

Treatment	Degradation rate (µg/g DW and day)	R^2
S – O	0.0023	0.866
S + O	0.0043	0.890
BM – O	0.0418	0.922
BM + O	0.0297	0.996

Methabenzthiazuron (MBTZ)

For MBTZ, the final percental dissipation in treatments S – O and S+ O were higher than in the treatments BM – O and BM + O (see Appendix A). The percental dissipation in S + O was higher compared to S - O. There was however no significant difference dissipation in BM – O and BM + O (see Appendix A).

A slight initial increase in MBTZ concentration is observed in the treatments with biomix (Figure 4-5). In the soil treatments, the initial dissipation in the highest during the study and slows down towards the end of the experiment. The dissipation rate in treatment S + O is slightly, but significantly higher compared to the dissipation rate in treatments S – O, whereas the addition of oil is not significantly increasing the dissipation rate in the biomix treatments (Figure 4-5).

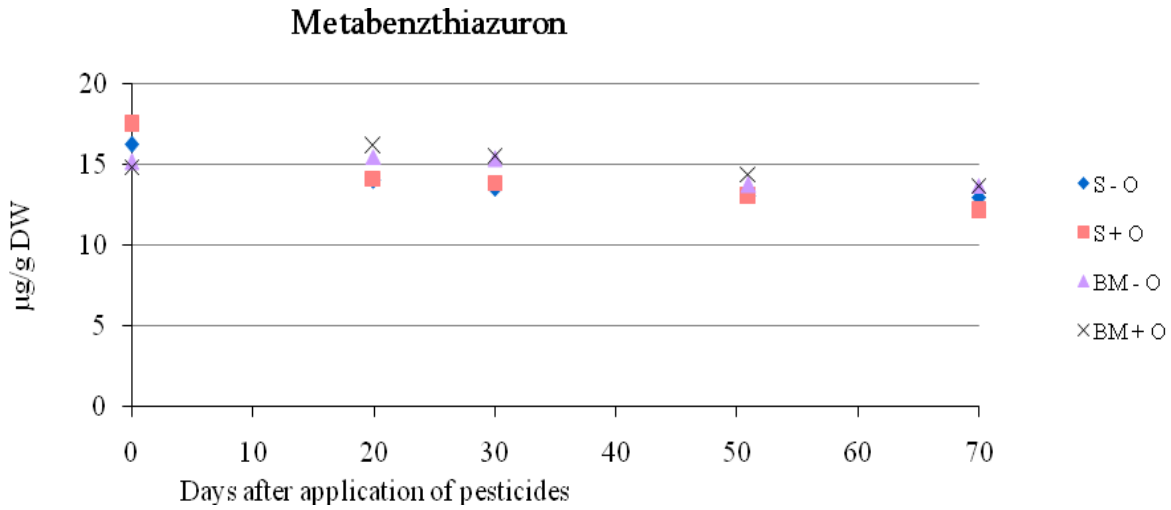


Figure 4-5 Concentration of methabenzthiazuron as mean values, n=3 in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) that were incubated at 30ºC.

Table 4-3 Average dissipation rate of methabenzthiazuron as mean values, n=3 in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) that were incubated at 30ºC.

Treatment	Degradation rate (µg/g DW and day)	R^2
S – O	0.0030	0.790
S + O	0.0048	0.888
BM – O	0.0028	0.867
BM + O	0.0033	0.994

Isoproturon (IP)

The dissipation of IP is significantly higher in the treatments with biomix compared to dissipation of IP in treatments with soil (Figure 4-6). The final dissipation is also higher

in the treatment with soil and oil, compared to plain soil. In the biomix however, there is no significant difference between the treatments with and without oil. The rate is higher in the treatment with biomix (Table 4-4) and oil, but due to an initial lag phase not present in the treatment without oil, the higher rate does not have any effect on the final dissipation.

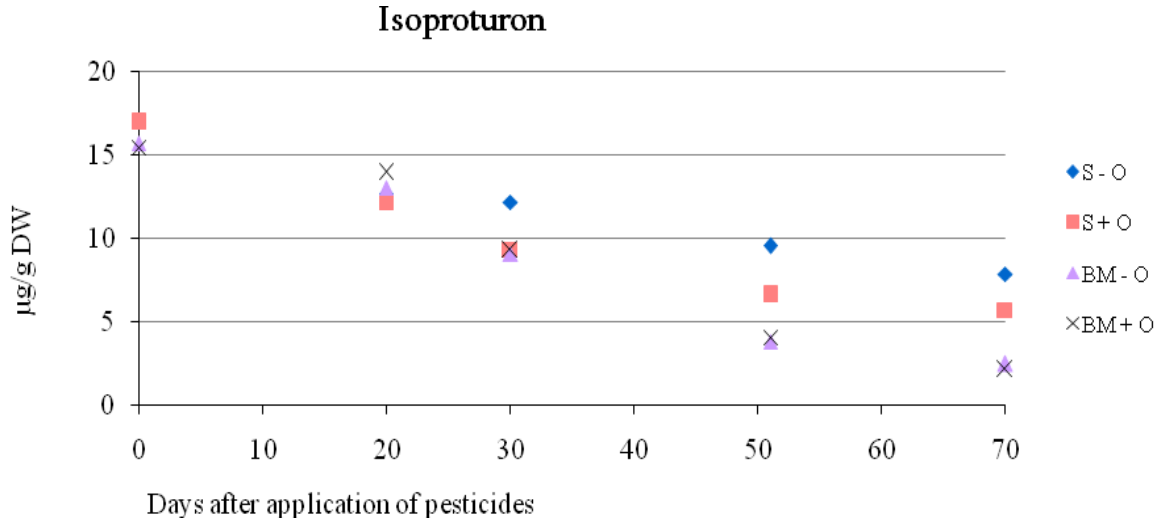


Figure 4-6 Concentration of isoproturon as mean values +- SD, n=3 in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) that were incubated at 30ºC.

Table 4-4 Average dissipation rate of isoproturon as mean values, n=3 in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) that were incubated at 30ºC.

Treatment	Degradation rate (µg/g DW and day)	R^2
S – O	0.0107	0.987
S + O	0.0162	0.987
BM – O	0.0342	0.985
BM + O	0.0381	0.998

Terbuthylazine (TBA)

The final dissipation is significantly higher in the treatments with biomix (Appendix A). There is no significant difference in the soil treatments with or without oil. In the treatment with biomix and oil, the dissipation is significantly lower with the addition of oil.

There is a lag phase in the treatment biomix with oil that in combination with a lower rate compared to the treatment biomix without oil, results in a lower final dissipation (see Table 4-5). The dissipation rate is generally higher in treatments with biomix compared to treatments with soil (Table 4-5).

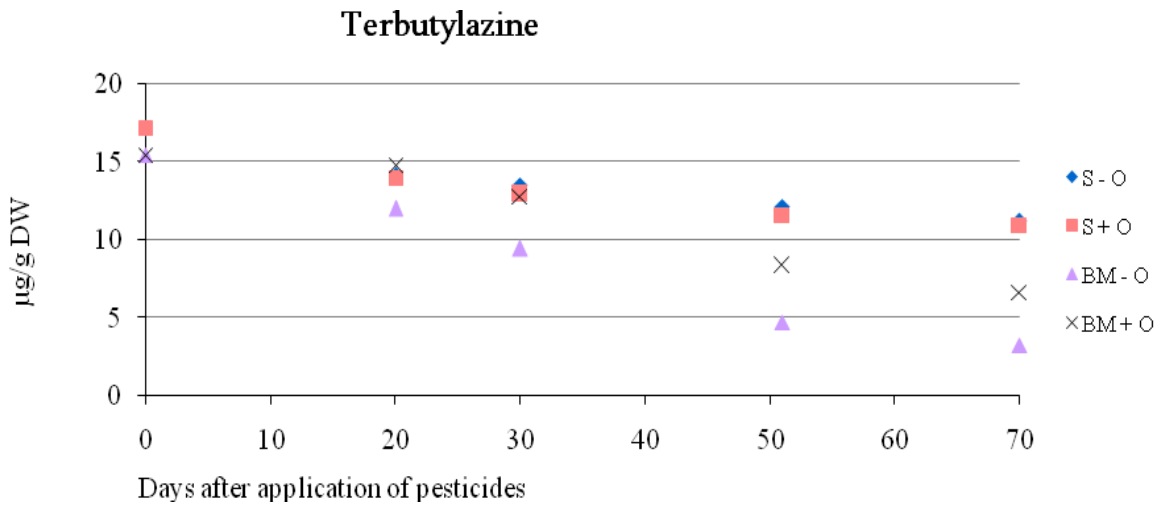


Figure 4-7 Concentration of terbuthylazine as mean values +- SD, n=3 in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) that were incubated at 30ºC.

Table 4-5 Average dissipation rate of terbuthylazine as mean values, n=3 in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) that were incubated at 30ºC.

Treatment	Degradation rate (µg/g DW and day)	R^2
S – O	0.0059	0.962
S + O	0.0063	0.938
BM – O	0,0241	0,974
BM + O	0,0167	0,989

Chlorpyrifos (CLP)

The highest final dissipation was observed in the treatment soil without oil (Appendix A). Biomix with oil added had a similar dissipation rate (Table 4-6), but due to an initial lag phase (Figure 4-8) the final dissipation is significantly lower compared to the treatment soil with oil. The treatments soil with oil and both biomix treatments are not significantly different, however the rates for soil with oil and biomix without oil are considerably lower compared to biomix with oil and soil without oil.

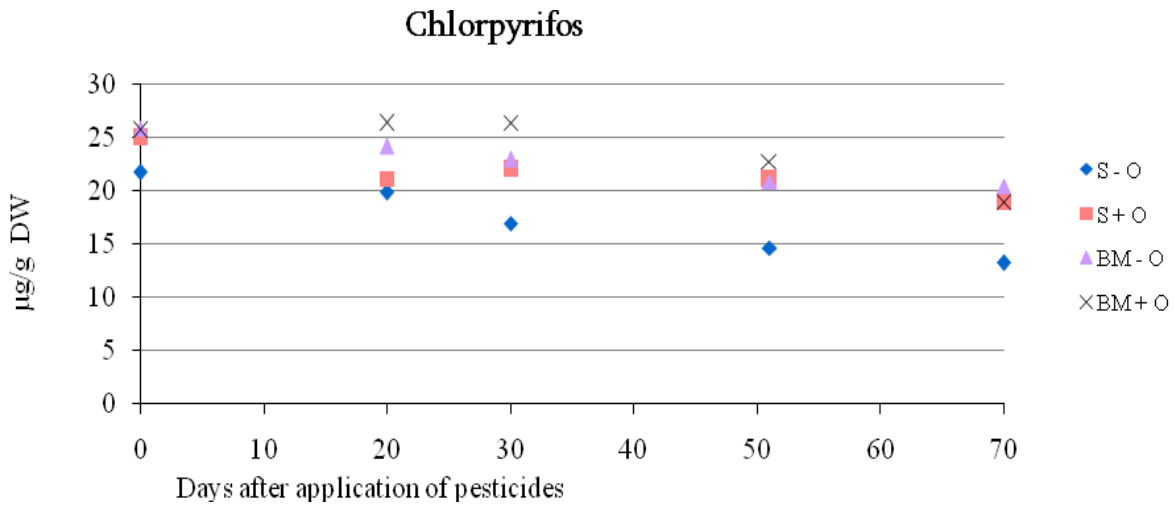


Figure 4-8 Concentration of chlorpyrifos as mean values +- SD, n=3 in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) that were incubated at 30ºC.

Table 4-6 Average dissipation rate of chlorpyrifos as mean values, n=3 in soil (S) and biomix (BM), with (+O) or without (-O) addition of oil (0.5 % V) that were incubated at 30ºC.

Treatment	Dissipation rate (µg/g DW and day)	R^2
S – O	0.0077	0.957
S + O	0.0033	0.818
BM – O	0.0037	0.971
BM + O	0.0082	0.992

4.5. Degradation of pesticides by pure MnP and vegetable oil

In the following sections the results from the *in vitro* experiment are presented individually for each pesticide. Selected graphs are presented in the Results section of the report, for complete time graphs for all pesticides and treatments, see Appendix B.

This experiment was designed to investigate whether pure MnP in combination with vegetable oil could increase degradation of pesticides *in vitro*, where H_2O_2 is added to initiate the reaction. Five controls were included in the experiment, one with oil and H_2O_2 and MnP, one with oil and H_2O_2, one with H_2O_2, one with MnP and H_2O_2, one with oil only, and one with MnP, H_2O_2, oil but without pesticides. For detailed information about the five treatments A-E, (see Table 3-2).

Isoproturon (IP)

The highest final dissipation was observed in the treatment with MnP, oil and H_2O_2. A high final dissipation was also observed in the treatment with MnP and without oil, but significantly lower compared to the treatment with both MnP and oil (Appendix B). As shown in (**Figure 4-9**), the dissipation was decreasing and appears to be absent from day 2 – 4, and after day 4 continuing to increase with the second addition of MnP. The initial dissipation rate was higher in treatment with MnP, oil and H_2O_2 compared to the treatment MnP and H_2O_2, where the degradation continues to increase through the experiment with an increased rate just after day 4.

Treatments B, C and E shows all significantly lower dissipation compared to treatment A and D, but there was no significant difference in final dissipation between treatment B, C and E. The slight increase in concentration at day 4 in the treatments without MnP is most likely due to the dilution from adding buffer instead of adding fresh enzyme.

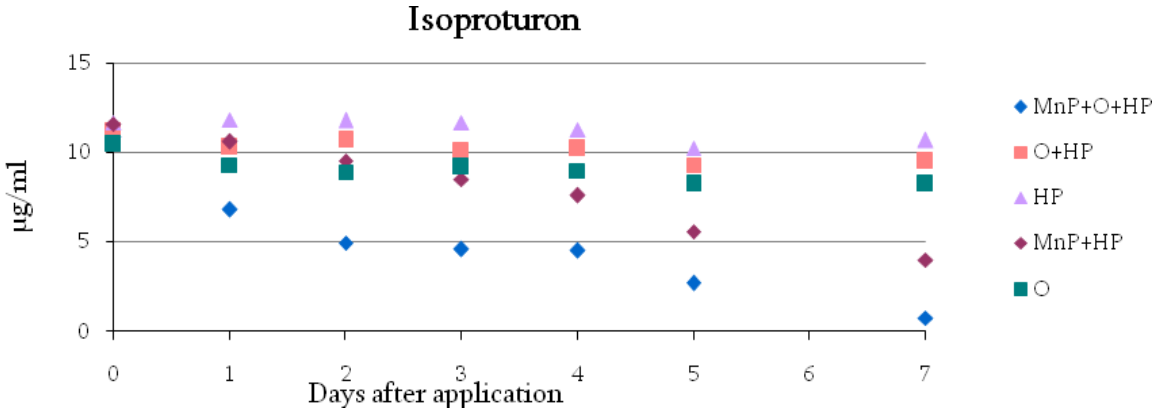


Figure 4-9 Concentration of isoproturon as mean values, n=3 with manganese peroxidase (MnP), rapeseed oil (O), hydrogen peroxide (HP) as described in legend and Na-Acetate buffer, Tween 20, MnSO4 at 37°C.

Terbutylazine (TBA)

The pattern for TBA was similar to IP, but only not as clear. As for IP the dissipation was initially high for the treatment MnP, oil and H_2O_2 (Figure 4-10), but for TBA an increase in concentrations was observed from day 2 to 4 instead of a lag phase. This increase in concentration was observed in all treatments with oil, which could indicate interference in the chromatogram. After day 4, where fresh MnP was added, TBA concentrations continued to decrease in the treatment with MnP, oil and H_2O_2 but not the other two treatments with oil.

Also the treatment with H_2O_2 shows similarity with the same treatment for IP. The high standard deviation for final dissipation of the treatment with H_2O_2 was due to a low dissipation until day 5, and samples from day 7 showed a very mixed dissipation (Appendix B). Only final dissipation of treatment MnP, oil and H_2O_2 and oil and H_2O_2, and MnP, oil and H_2O_2 and oil were significantly different.

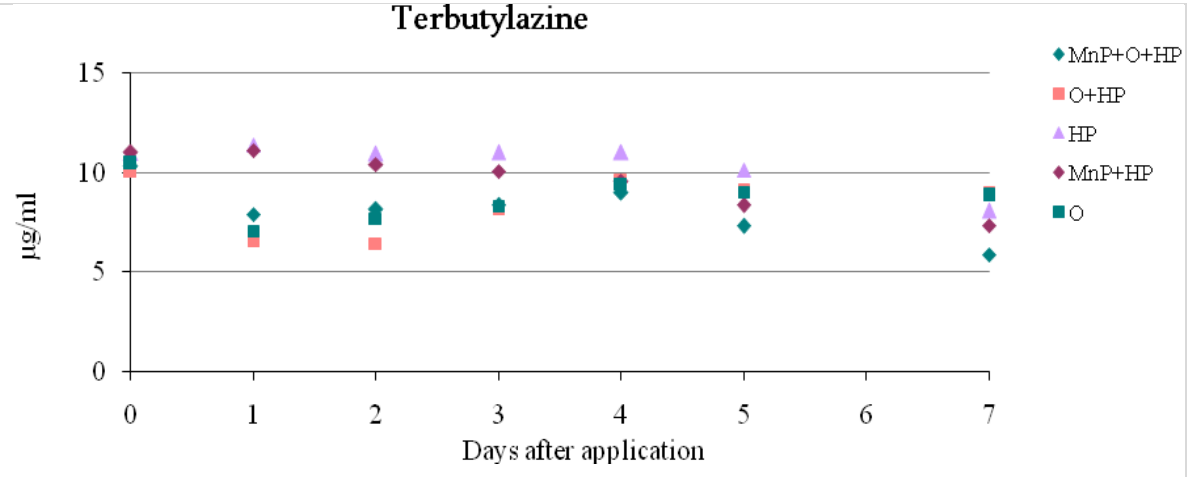


Figure 4-10 Concentration of TBA as mean values, n=3 with manganese peroxidase (MnP), rapeseed oil (O), hydrogen peroxide (HP) as described in legend and Na-Acetate buffer, Tween 20, MnSO4 and incubated at 37°C.

Chlorpyrifos (CLP)

For CLP, the pattern was very different compared to TBA and IP. Here the treatment with oil and H_2O_2 and the treatment with oil had higher final dissipation compared to treatment MnP, oil and H_2O_2 and MnP and H_2O_2 (Figure 4-11). Only the treatment with oil and H_2O_2 had significantly different final dissipation from the treatments H_2O_2 and MnP and H_2O_2. The difference compared to IP and TBA is that a lower final dissipation was observed in treatment MnP, oil and H_2O_2 and MnP, and H_2O_2 for CLP.

In the treatment with oil only, a similar increase in concentration as for TBA could be observed from day 2 to 4, but not in the other treatments with oil.

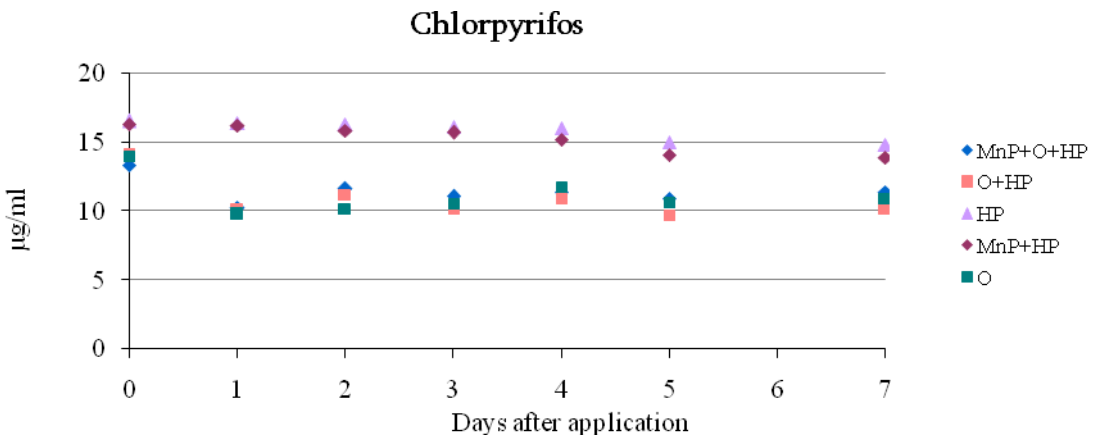


Figure 4-11 Concentration of chlorpyrifos as mean values, n=3 with manganese peroxidase (MnP), rapeseed oil (O), hydrogen peroxide (HP) as described in legend and Na-Acetate buffer, Tween 20, MnSO4 that kept at 37°C.

Bentazone (BZ) and Methabenzthiazuron (MBTZ)

No reliable results could be obtained from the *in vitro* experiment for BZ and MBTZ due to interference from degradation products from oil and pesticides. In the chromatograms, three reoccurring peaks were detected and studied closer, due to the risk of interference with measured pesticides.

At 10.9 min, a peak that interferes with BZ was encountered. This peak was found normally when oil was present and occurred already at day 0. The peak increased to day 3 or 4, where it again increased to day 5 and 7. Due to low separation during BZ analysis, this peak was partly or entirely covered by the BZ peak, and was therefore most likely interfering with BZ results. One peak with same retention time as MBTZ (around 11.2 min) occurred in all treatments with oil. Normally it appeared at time 1 or 2 days, and increased steadily until day 7. This peak was not at all detected in the chromatogram for MBTZ, and therefore it was assumed that this peak was covered by the MBTZ peak and might interfere with the result. Graphs are presented in Appendix B.

5. DISCUSSION AND CONCLUSIONS

The objectives with this experiment were to investigate whether rapeseed oil could improve the degradation rate for a mixture of pesticides both in a biomix and *in vitro* by pure MnP, and whether there was any variation in how this influence dissipation of different pesticides.

The results from the degradation experiment vary. For three of the pesticides (BZ, IP and TBA) the average final dissipation in the biomix treatments are significantly higher compared to the soil treatments. The dissipation of MBTZ is significantly higher in the soil treatments compared to the average final dissipation in the biomix treatments. The dissipation of CLP is significantly higher in soil without oil added, compared to soil with oil and the biomix treatments.

The results from the addition of oil also vary between pesticides. An increased final dissipation from the oil can only be observed for the soil treatments, where the addition of oil increases dissipation for three of the pesticides (MBTZ, IP and CLP). In biomix without oil, about 14% of the initial amount of IP remained which is consistent with the results in previous studies (von Wirén-Lehr, Castillo et al. 2001). From a previous study, CLP has been measured with a half-life of 47 days in biomix without unsaturated fatty acids added (Coppola, Castillo et al. 2006). In this study, the final dissipation in similar biomixes was considerably lower, 20% after 70 days. For BZ and TBA there is no significant difference between oil added and no oil added to the soil treatments. The addition of oil has no effect (BZ, MBTZ, IP and CLP), or a negative effect on dissipation in biomix (TBA). However, the lack of dissipation of MBTZ in the biomix without oil is consistent with previous studies (Castillo and Torstensson 2007).

As for the *in vitro* degradation study, the results vary between the different pesticides. A similar pattern is observed for IP and TBA, where the dissipation pattern is most distinct for IP. The final dissipation of IP is significantly higher in the treatment with MnP, oil and H_2O_2 compared to the treatment with MnP and H_2O_2. A final dissipation of IP much lower compared to treatment MnP, oil and H_2O_2 and MnP and H_2O_2 is observed in the treatments with oil and/or H_2O_2. The pattern is similar for TBA but with less significant differences, partly because of larger standard deviations.

For both IP and TBA, no dissipation occurs between day 2 and day 4. A more frequent addition of MnP could continue the dissipation during the experiment. A complete dissipation of IP is very likely, and also TBA could have had a significantly higher final dissipation. The measured dissipation of CLP *in vitro* with presence of MnP with

unsaturated fatty acids have in previous studies (Coppola, Castillo et al. 2006) been reported at twice the rate compared to this study. IP on the other hand shows similar dissipation compared to previously performed studies with unsaturated fatty acids (Castillo, von Wirén-Lehr et al. 2001).

In the in vitro experiment, a rebound in concentration could be observed for TBA in the treatments with oil, but also for in the treatment with oil only for CLP. This pattern cannot be observed for IP, and in the other oil treatments for CLP. Due to the steady increase in concentration between day 2 and 4, insufficient mixture of vials is not a likely explanation. A rapid dissipation is observed between day 0 and day 1, after which the TBA is starting to increase in concentration. No potentially interfering peaks from oil can be observed in the chromatograms. However, interference from a degradation product of TBA, with the same retention time as TBA, cannot be excluded. In a degradation study of PAHs with the addition of rapeseed oil, the vegetable oil alone was found to increase dissipation (Pizzul, Castillo et al. 2007). If the oil alone degraded TBA, the degradation product would be different compared to the degradation product from degradation with MnP, since the increase in concentration was only observed in the treatments with oil.

The phenoloxidase activity was higher in soil with oil compared to soil without oil, however there was no apparent difference between biomix with and without oil. In studies of rapeseed oil influence on degradation of PAH:s (Pizzul, Castillo et al. 2007) 0,1 % v/v of rapeseed oil was found to promote dissipation of PAH whereas 1% v/v of rapeseed oil was found to inhibit enzymatic activity. It is possible that also the amount used in this work, 0.5% v/v, could inhibit production of enzymatic activity. But with a general lack of phenoloxidase activity no conclusions can be made from this. This is however contradicted by the respiration results, where the respiration is slowing down in the biomix treatment with oil towards the end of the experiment. Furthermore, the lack of correlation between accumulated respiration and phenoloxidase activity only indicates that there are several microbial organisms active in the soil and biomix, and the active ligninolytic fungi does not constitute the majority of respiratory organisms. If the respiration is inhibited by oil, the inhibited microorganisms are most likely not related to the production of MnP.

Except for the proposed increased dissipation with lipid peroxidation, the oil could also increase the solubility of the pesticides. The addition of oil has however no effect on the initial extraction efficiency. It is unknown is whether the extraction efficiency changed during the experiment and whether the oil could be part of the reason.

Since the pesticides were added to the biomix at a relatively high concentration, this could also be a potential cause for the inhibition of the growth of ligninolytic fungi. After half of the experimental time, the phenoloxidase activity increased again. This could be explained by the microorganisms first being inhibited to some degree by the high pesticide concentration, and after some time, when the pesticide concentration had decreased by non-ligninolytic degradation mechanisms, the conditions were favourable for ligninolytical fungi.

With no method available to measure activity of only MnP, the phenoloxidase activity was regarded as a good estimate. Phenoloxidase activity includes mainly MnP, but interference from LiP and L occurs. Since these two enzymes also degrade recalcitrant substances, it is not possible to determine which of these enzymes that contributes to the dissipation of the pesticides (Castillo, Ander et al. 1997). With the lack of phenoloxidase activity in combination with a significant dissipation of the pesticides, it is likely that also other ligninolytical enzymes and metabolical processes are involved in the dissipation of these pesticides.

The *in vitro* experiment for BZ presents an initial decrease in concentration of BZ that towards mid-experimental time is stabilising and followed by an increase in concentration. A peak with similar retention time as BZ was observed in the chromatogram. This peak has a retention time similar to BZ and as this compound increases in concentration, the interference with the BZ peak is increasing towards the end of the study.

At an initial evaluation this did not seem to be the case with the increasing concentrations of MBTZ, since there were no other peaks than the MBTZ peak on the chromatogram. However, after studying the chromatogram for other pesticides, a peak with the exact same retention time as MBTZ was observed in the chromatograms from the treatments including oil.

Due to these peaks, the dissipation results of BZ and MBTZ from the degradation experiment should be interpreted carefully. This might also be an explanation why a lower degradation of BZ is observed compared to previous studies.

Further, with the extraction efficiency of over 100% and the reoccurring peaks from oil, detected in the *in vitro* experiment, it can be questioned whether the results for MBTZ are reflecting an actual lower dissipation in biomix or whether the lower dissipation in biomix is related to interference during analysis.

Due to this interference the results from the BZ and MBTZ analyses are not reliable for interpretations. A new analytical method is needed for these compounds but was not possible to develop within the scope of this work.

5.1. Summary and conclusions

The main results from these studies are summarized below:

- The effect from the addition of oil varies from promoting degradation to inhibiting degradation depending on pesticide. The lack of correlation with phenoloxidase activity could indicate that degradation mechanisms other than ligninolytic enzymes were prevailing or even dominant in this study.

- The microbial respiration was higher in biomix than in soils. The addition of oil had an inhibitory effect in the biomix.

- The phenoloxidase activity was higher in the biomix compared to the soil. The addition of oil did not increase the enzymatic activity.

- In the *in vitro* experiments the addition of oil enhanced the degradation of IP by MnP, but no positive effect was observed for CLP, TBA, BZ and MBTZ. However, no enhancement of IP degradation was obtained in the biomix in the presence of rapeseed oil.

- The interference peak in the chromatograms which increased over the incubation time could a product from vegetable oil being degraded in presence of of MnP could possibly indicate lipid.

- The initial method for the HPLC analysis were developed so all five pesticides could be detected within a reasonable time frame. However, with the oil-fractions peaking around MTBZ and BZ, and the fact that CLP had to be analysed separately, a new method also for the other four pesticides should be developed for further studies. The new method for the four pesticides should separate the peaks around BZ and MBTZ further and not take CLP into account.

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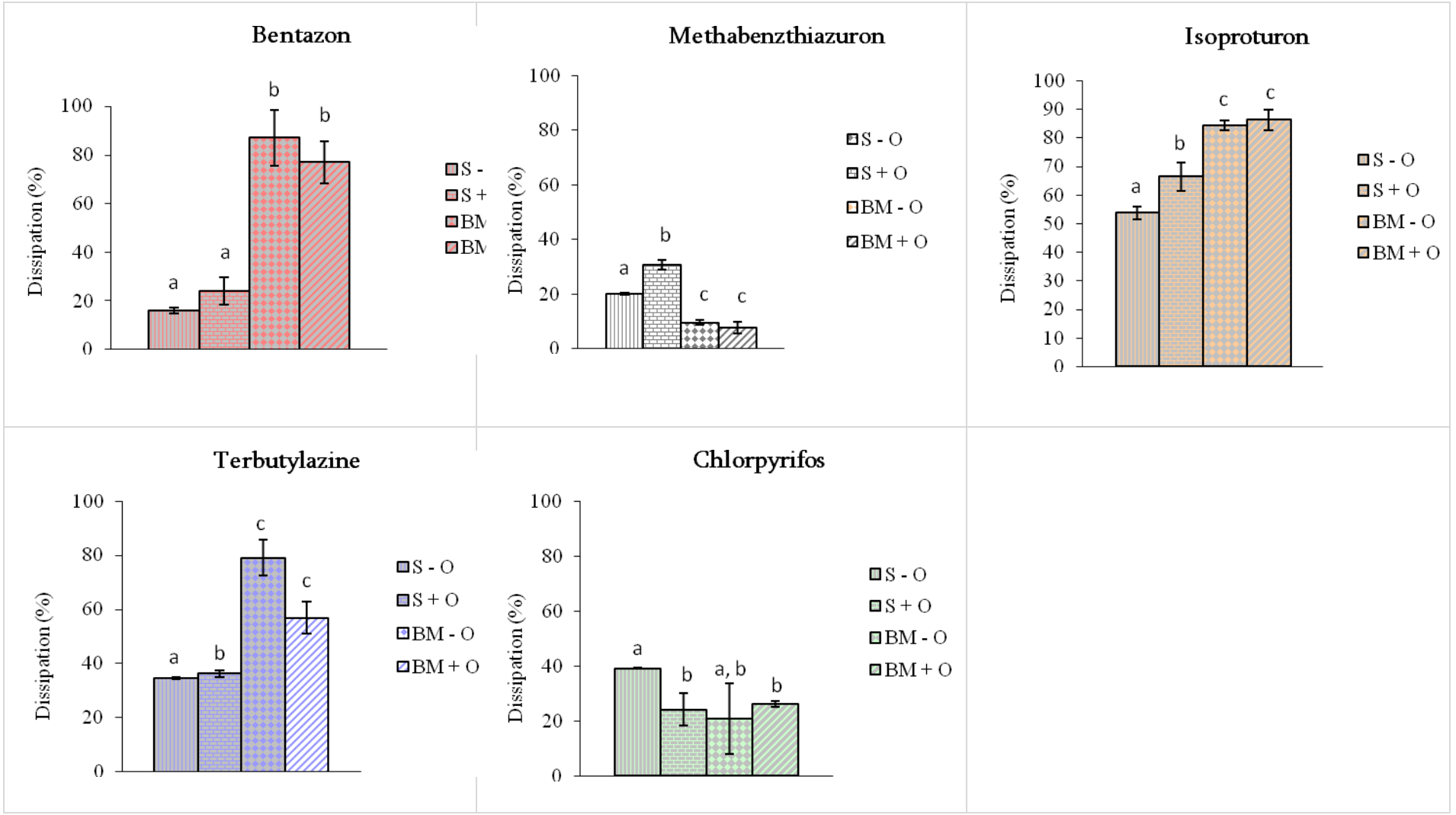
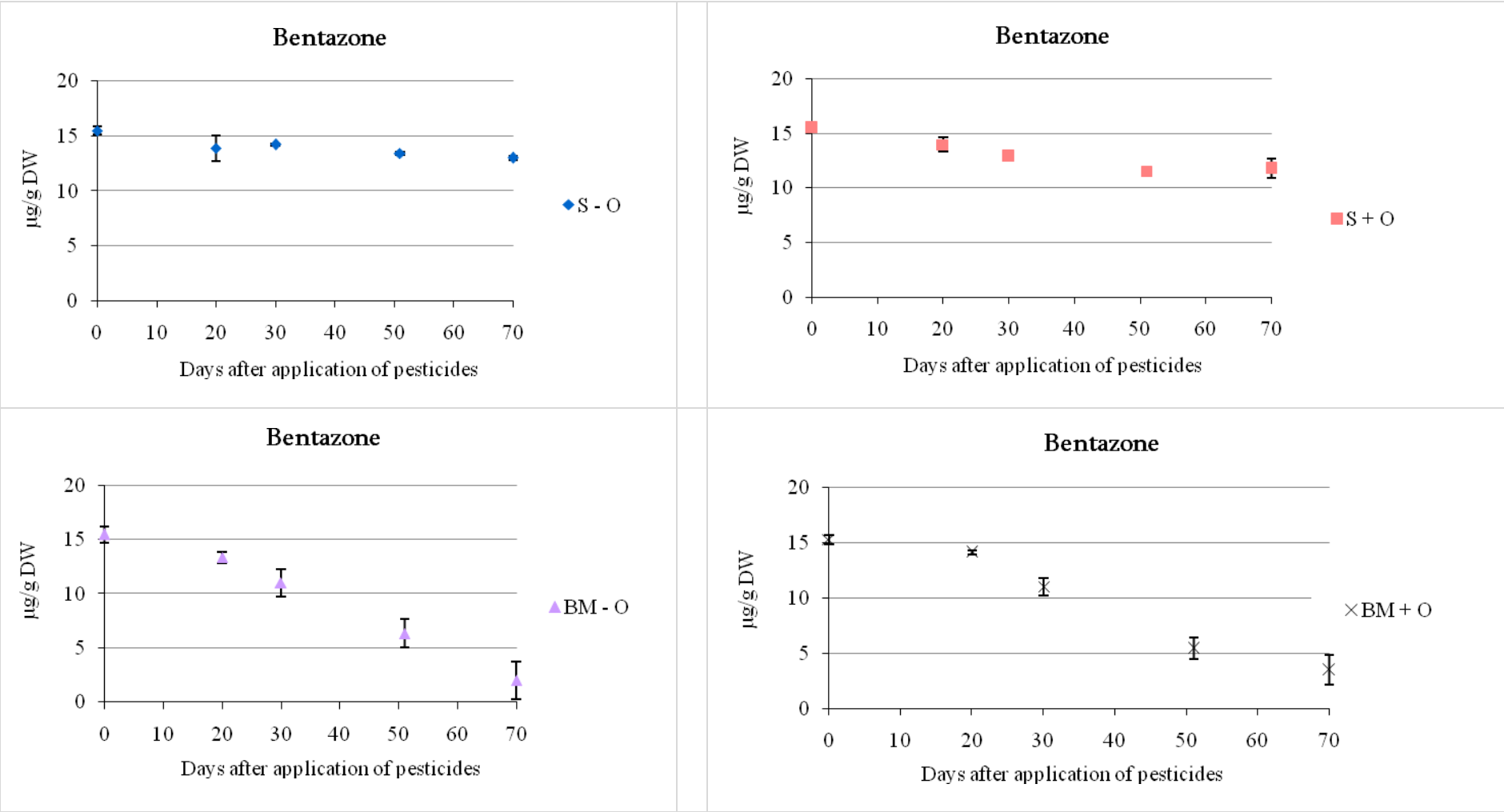
Further I'd like to thank my friend Kattis, fellow master student and now colleague, for pushing me. Finally I'd like to thank my parents, sister and grandmother for their great support during my time at SLU.

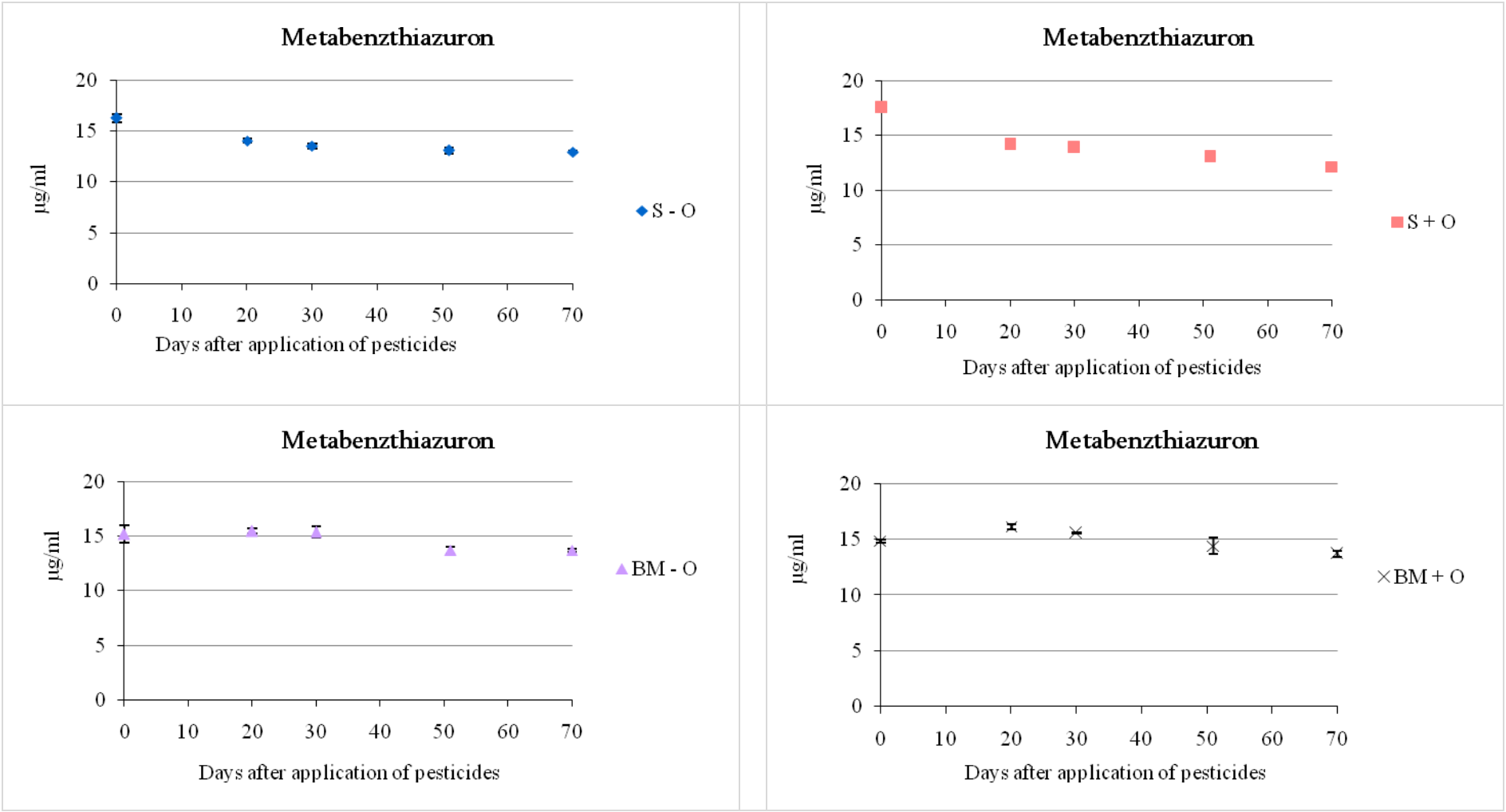
Appendix A
Graphs: Degradation study



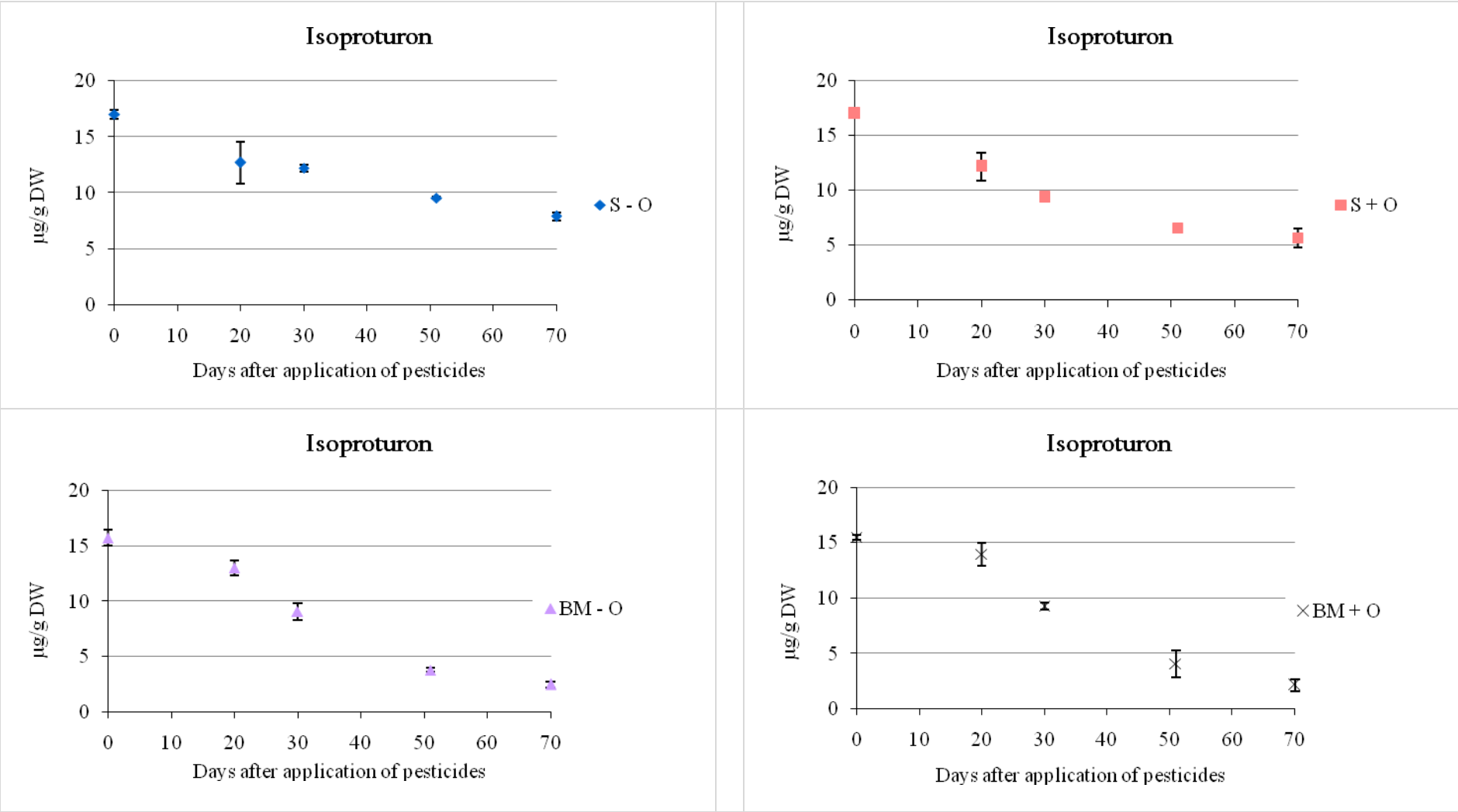
Figures show total dissipation of Bentazone, Methabenzthiazuron, Isoproturon, Terbutylazine and Chlorpyrifos after 60 days in respective treatment; Soil without oil (S – O). Soil with oil (S + O), Biomix without oil (BM – O) and Biomix with oil (BM + O), where letters a, b, c indicates whether the treatments are significantly different from eachother.



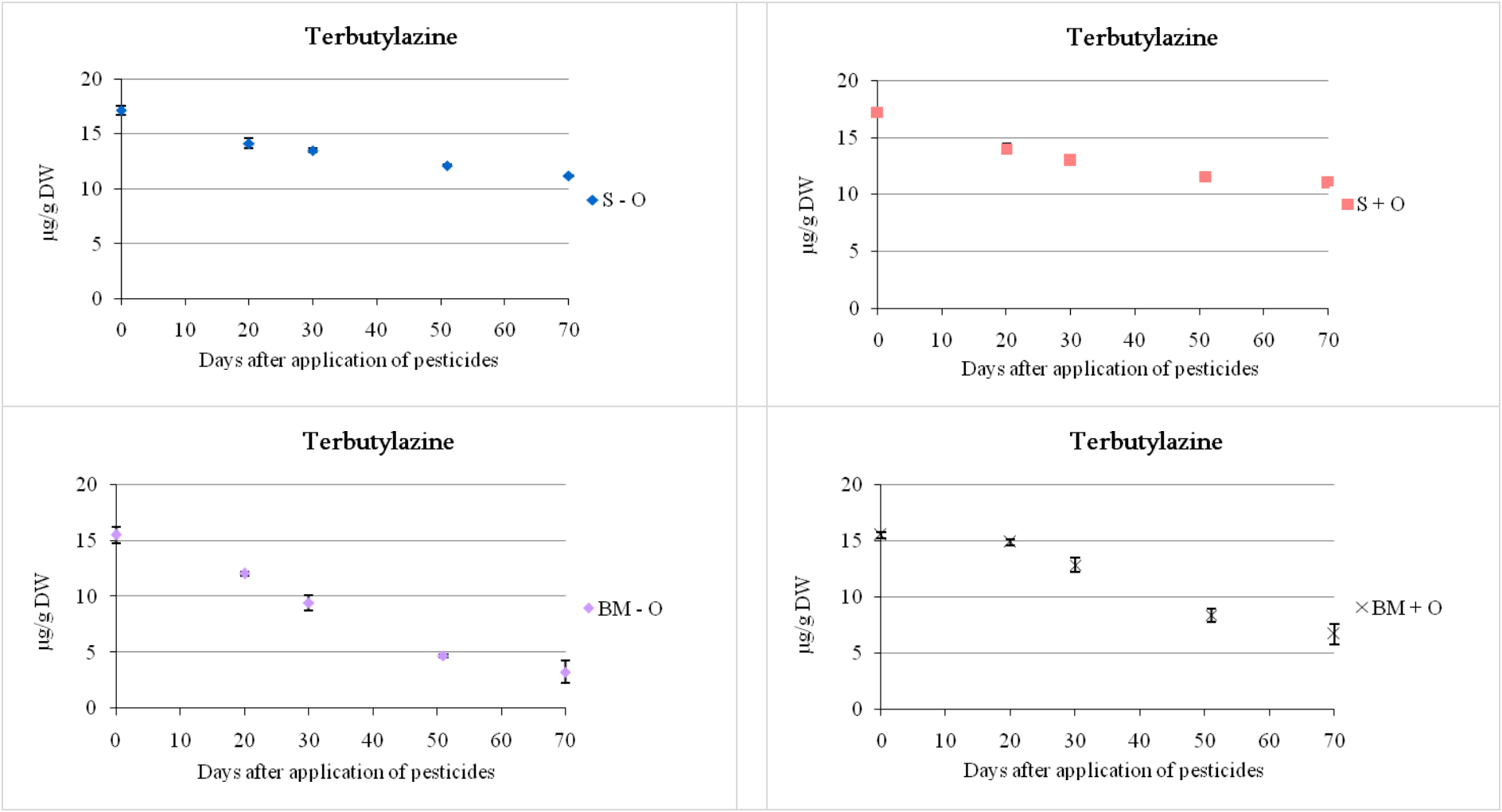
Figures show total concentration of Bentazone at day 0, 20, 30 50 and 70 days in respective treatment ± SD; Soil without oil (S – O). Soil with oil (S + O), Biomix without oil (BM – O) and Biomix with oil (BM + O).



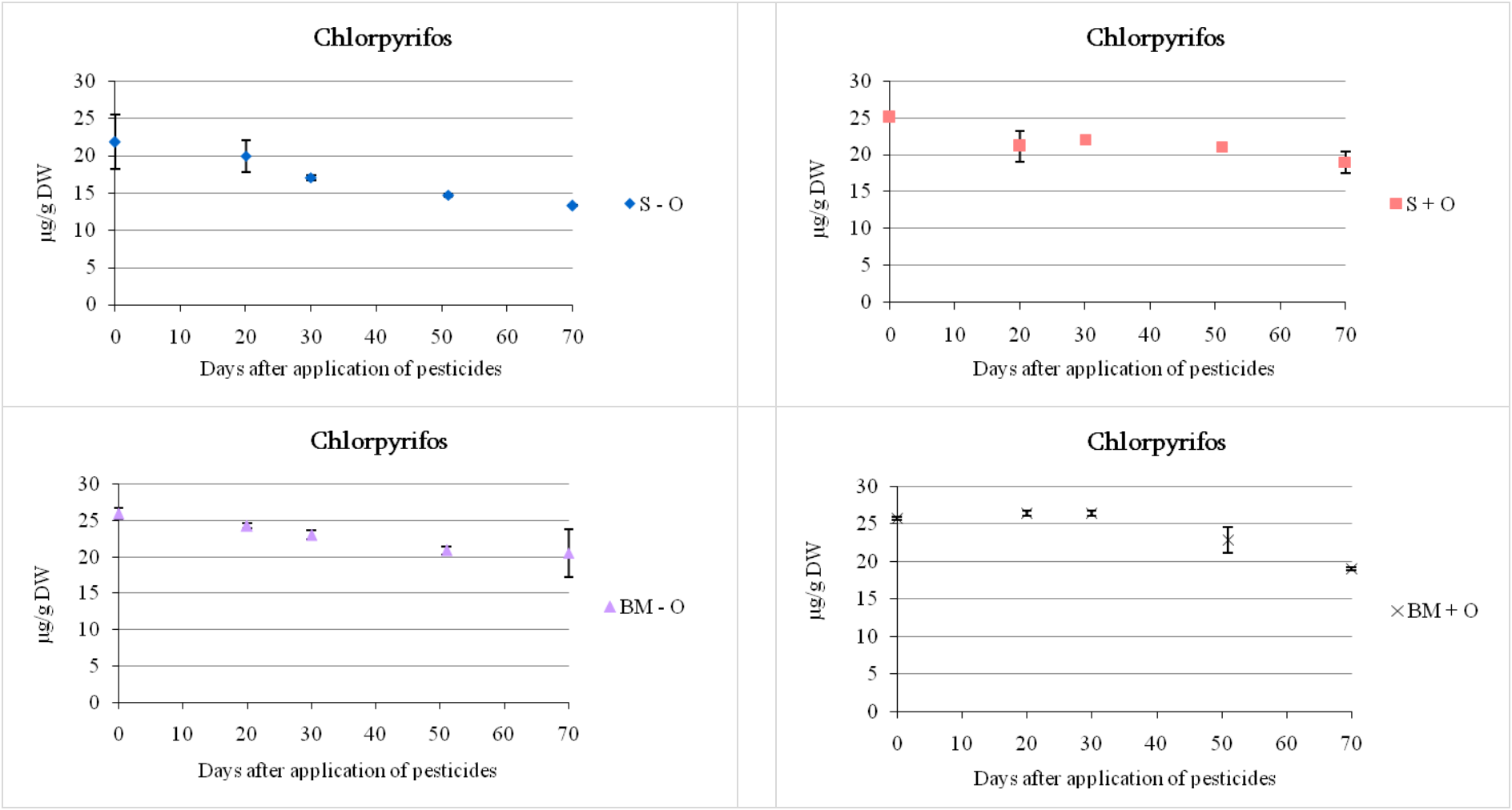
Figures show total concentration of Methabenzthiazuron at day 0, 20, 30 50 and 70 days in respective treatment ± SD; Soil without oil (S – O). Soil with oil (S + O), Biomix without oil (BM – O) and Biomix with oil (BM + O).



Figures show total concentration of Isoproturon at day 0, 20, 30 50 and 70 days in respective treatment ± SD; Soil without oil (S – O). Soil with oil (S + O), Biomix without oil (BM – O) and Biomix with oil (BM + O).

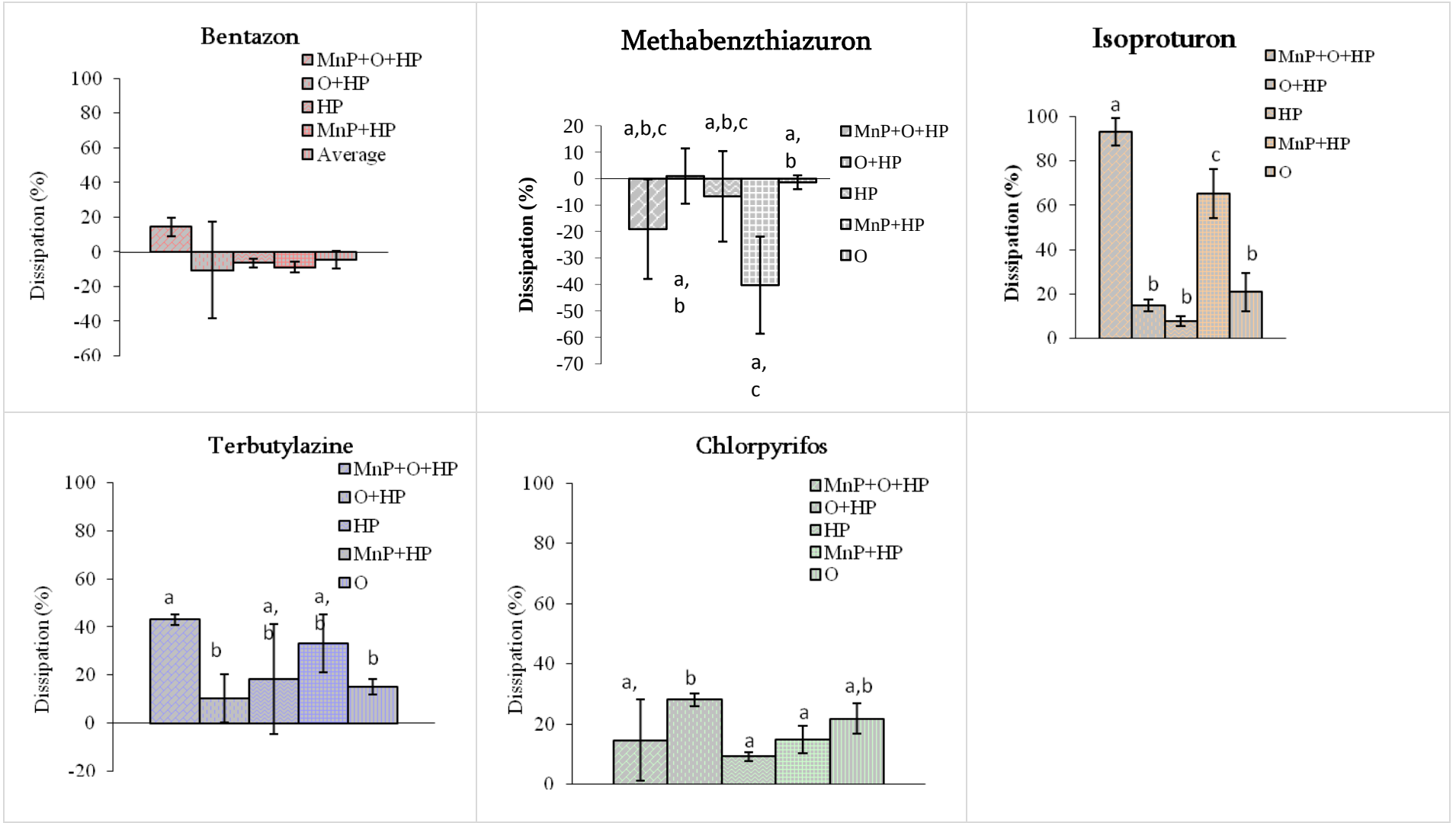


Figures show total concentration of Terbutylazine at day 0, 20, 30 50 and 70 days in respective treatment ± SD; Soil without oil (S – O). Soil with oil (S + O), Biomix without oil (BM – O) and Biomix with oil (BM + O).

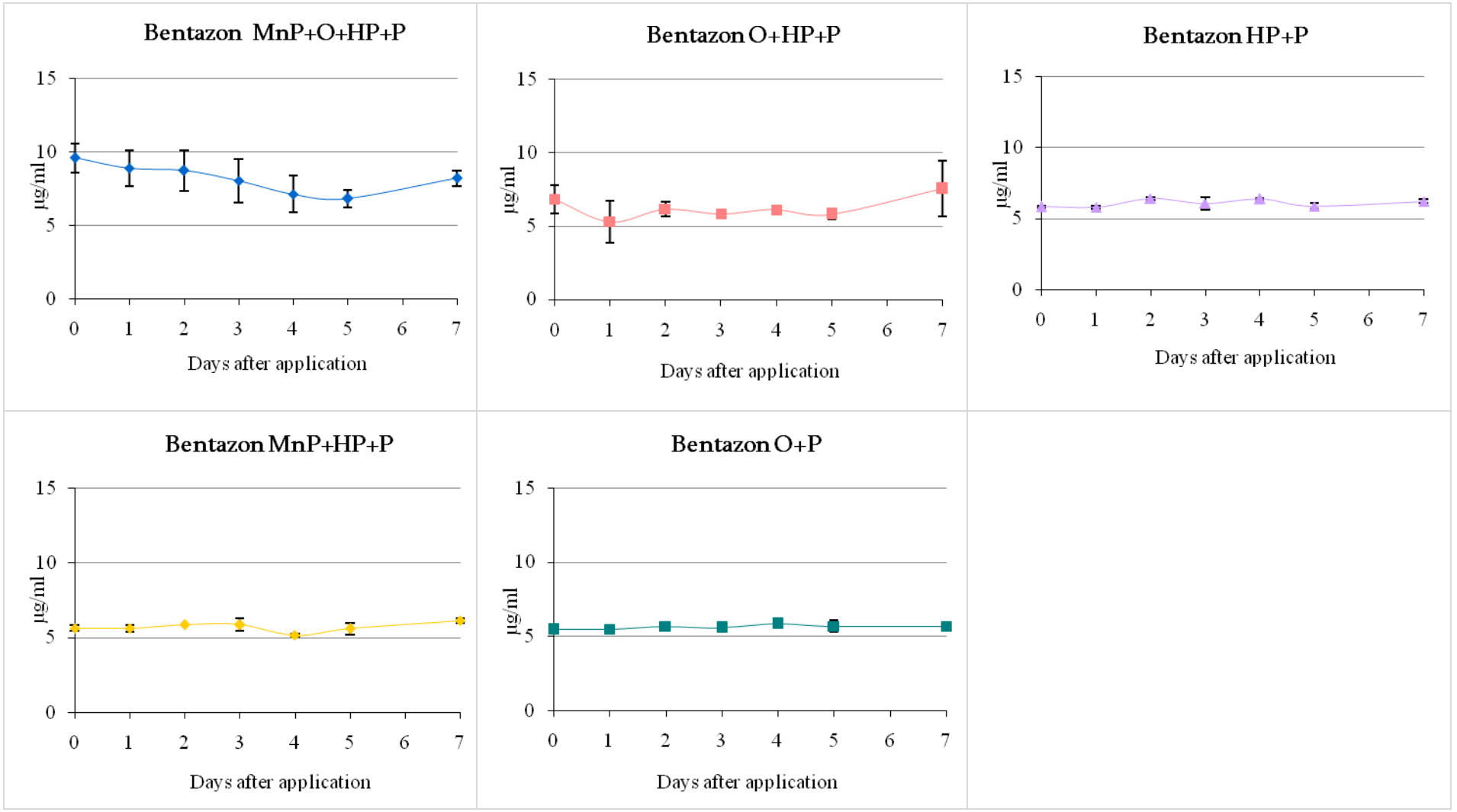


Figures show total concentration of Chlorpyrifos at day 0, 20, 30 50 and 70 days in respective treatment ± SD; Soil without oil (S – O). Soil with oil (S + O), Biomix without oil (BM – O) and Biomix with oil (BM + O).

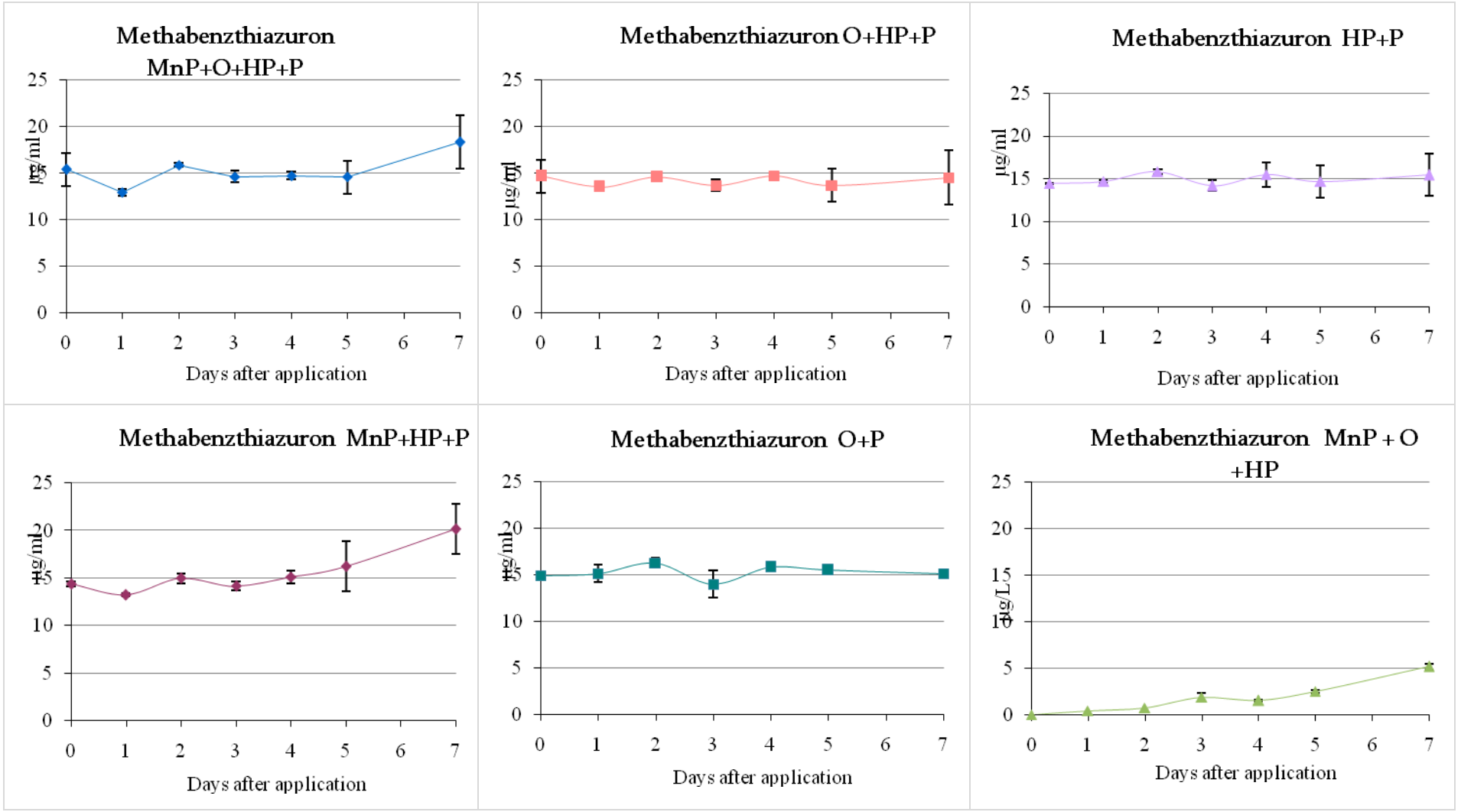
Appendix B
Graphs: In Vitro study



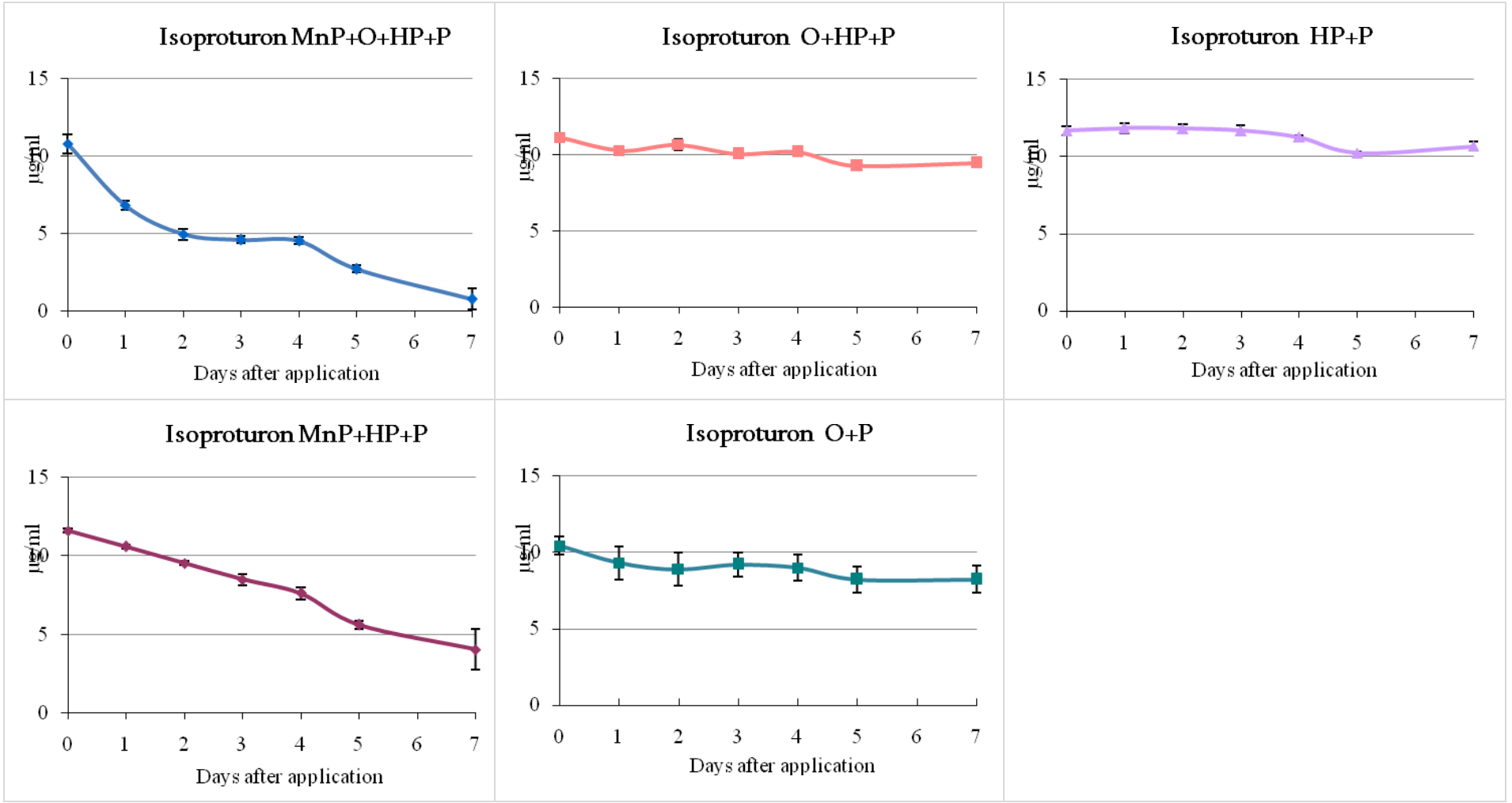
Figures show total dissipation of Bentazone, Methabenzthiazuron, Isoproturon, Terbutylazine and Chlorpyrifos after 7 days in respective treatment ± SD; with the addition of Manganese Peroxidase (MnP), Oil (O), Hydroge Peroxide and Pesticide mixture (P) as presented, where letters a, b, c indicates whether the treatments are significantly different from eachother.



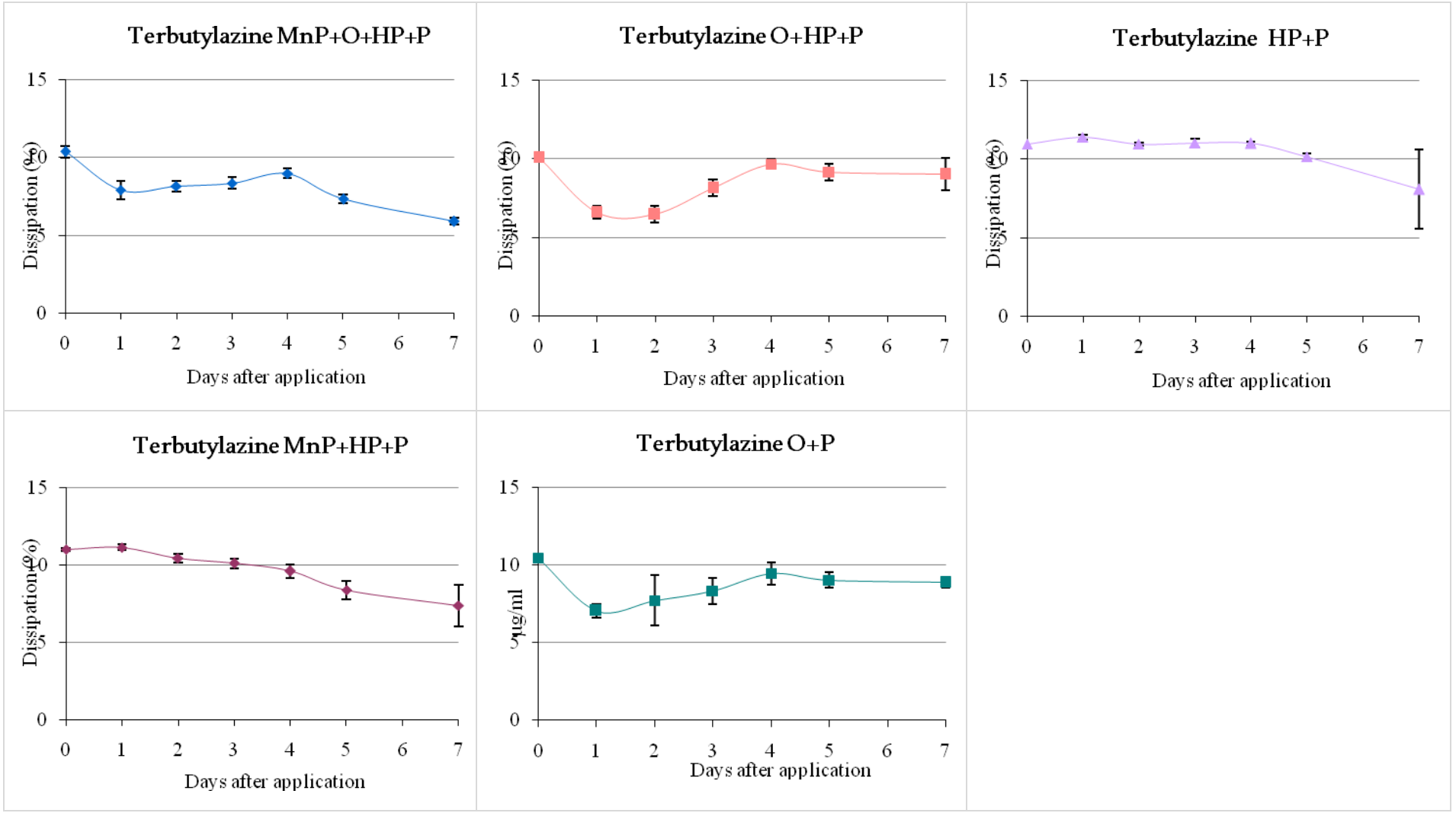
Figures show total concentration of Bentazone at day 0, 20, 30 50 and 70 days in respective treatment ± SD; with the addition of Manganese Peroxidase (MnP), Oil (O), Hydroge Peroxide and Pesticide mixture (P) as presented.



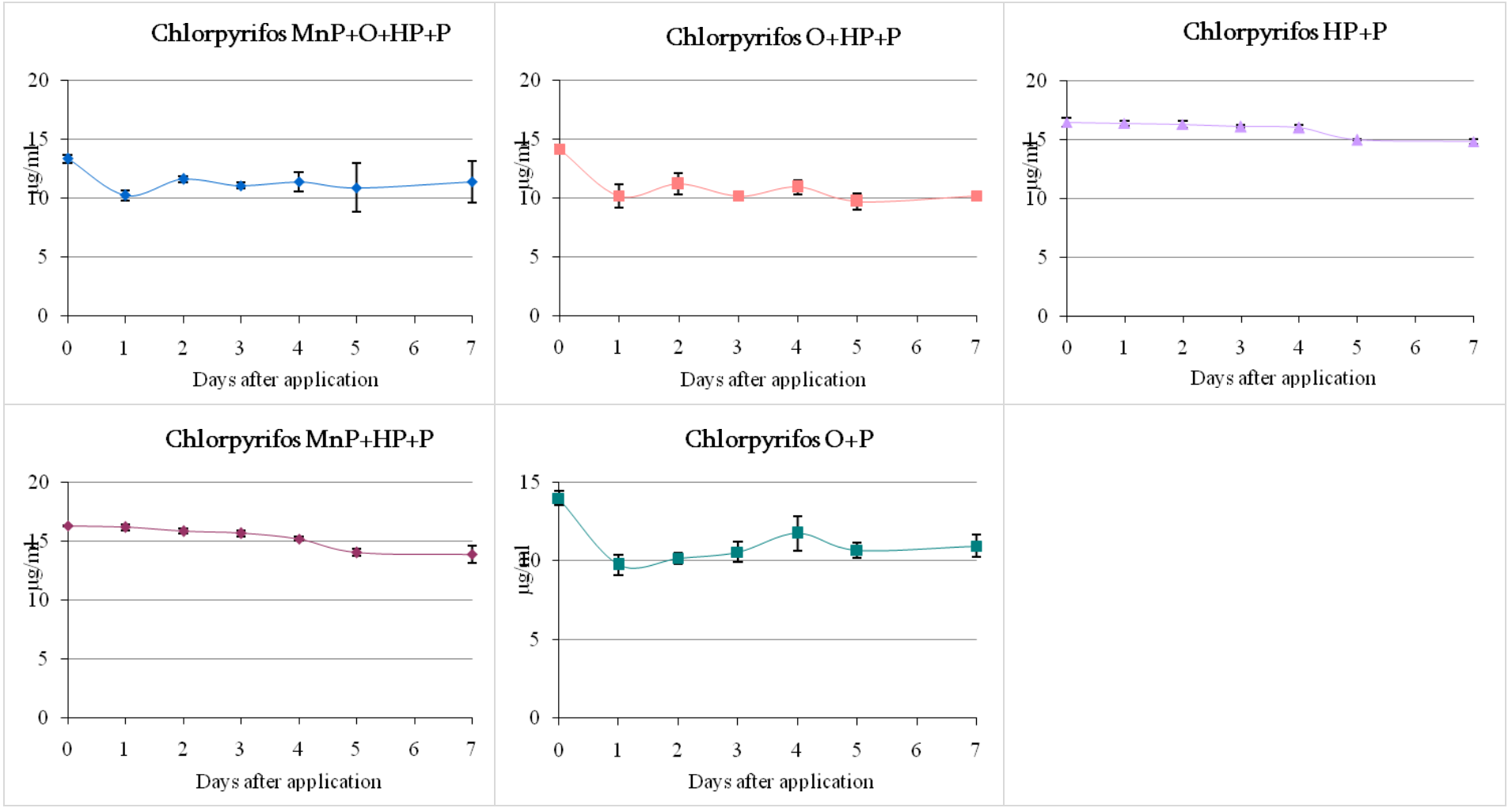
Figures show total concentration of Metabenzthiazuron at day 0, 20, 30 50 and 70 days in respective treatment ± SD; with the addition of Manganese Peroxidase (MnP), Oil (O), Hydroge Peroxide and Pesticide mixture (P) as presented.



Figures show total concentration of Isoproturon at day 0 – 7 of the in vitro test in respective treatment ± SD; with the addition of Manganese Peroxidase (MnP), Oil (O), Hydroge Peroxide and Pesticide mixture (P) as presented.



Figures show total concentration of Terbutylazine at day 0 – 7 of the in vitro test in respective treatment ± SD; with the addition of Manganese Peroxidase (MnP), Oil (O), Hydroge Peroxide and Pesticide mixture (P) as presented.



Figures show total concentration of Chlorpyrifos at day 0 – 7 of the in vitro test in respective treatment ± SD; with the addition of Manganese Peroxidase (MnP), Oil (O), Hydroge Peroxide and Pesticide mixture (P) as presented.